Wnt, Hedgehog and Notch signalling in relation to tapeworm anteroposterior polarity and segmentation

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by

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I, Francesca Louise Stark Jarero confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Tapeworms are a group of parasitic flatworms whose uniquely segmented body plan has raised questions regarding their anteroposterior polarity and homology to other animals for more than a century. The Wnt, Hedgehog and Notch pathways are conserved developmental signalling pathways involved in embryonic patterning and segmentation in the Metazoa. In this thesis, these three pathways were explored in a tapeworm for the first time. Spatial expression patterns for components belonging to each of the pathways were investigated in the mouse bile duct tapeworm, *Hymenolepis microstoma*. Wnt pathway factors showed polarised, conserved patterns of expression of 'posterior' ligands (*Wnts*) and 'anterior' inhibitors (*Sfrp*s). The expression of Wnt inhibitors, in particular, identified the true developmental anterior of *H. microstoma* larvae, answering a previously unresolved question of tapeworm development. In adult worms, expression patterns of these Wnt inhibitors confirmed the scolex (a region of the worm presumed to be anterior) as truly anterior. Expression of inhibitors and 'posterior' *Wnts* during strobilation shows polarised patterns along the anteroposterior boundaries of individual segments. These expression patterns indicate that the role of the Wnt pathway in specifying anteroposterior axes is conserved amongst tapeworms. Furthermore, a tapeworm-specific *Wnt11* paralog is hypothesised to have taken on a novel function during adult development, likely acting as the effector of strobilation. The expression of *Hmic-Wnt11a* is highly restricted between the neck and strobila and defines a newly recognised region of the adult body, dubbed the 'transition zone'. Hedgehog signalling in adult *H. microstoma* is linked with the nervous system (as it is in free-living flatworms). During larval development Hedgehog shows canonical specification of the midline. Notch signalling was found to be involved in a number of developmental processes. The expression pattern of *Hmic-Notch1* was of particular interest as it showed polarised posterior expression within all segments. Finally, this study developed single and double fluorescent *in situ* hybridisation techniques using tyramide signal amplification and lead the first steps towards an *in vivo* approach to RNA interference method in *H. microstoma*.

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Chapter I

Introduction

1.1 Parasitic flatworms

Flatworms (Platyhelminthes) are a highly diverse phylum of bilaterally symmetrical, dorsoventrally flattened acoelomate worms that include both free-living and parasitic groups. Most described flatworm species are obligatory parasites belonging to the monophyletic clade the Neodermata, or “new skin”, united by the presence of a syncytial neodermis, or tegument. The neodermatans represent one of the most successful groups that have shifted to a parasitic lifestyle and include the Monogenea, Trematoda (flukes) and Cestoda (tapeworms).

Tapeworms are obligate parasites, highly cosmopolitan with complex lifecycles that (mostly) infect more than one host species across several different life stages (Fig. 1.1). There are approximately 6000 species of tapeworms with many uniquely adapted to a single final host species. Infections in humans are responsible for a huge burden on public health as the causative agents of two Neglected Tropical Diseases (NTDs): echinococcosis and taeniasis/cysticercosis. The most important forms of these NTDs (in terms of their impact on human health) are caused by infections with three species: *Echinococcus granulosus*, *E. multilocularis* and *Taenia solium*. Infections can lead to severe morbidity and even death and are responsible for an estimated 3.72 disability adjusted life years (Torgerson et al., 2015).

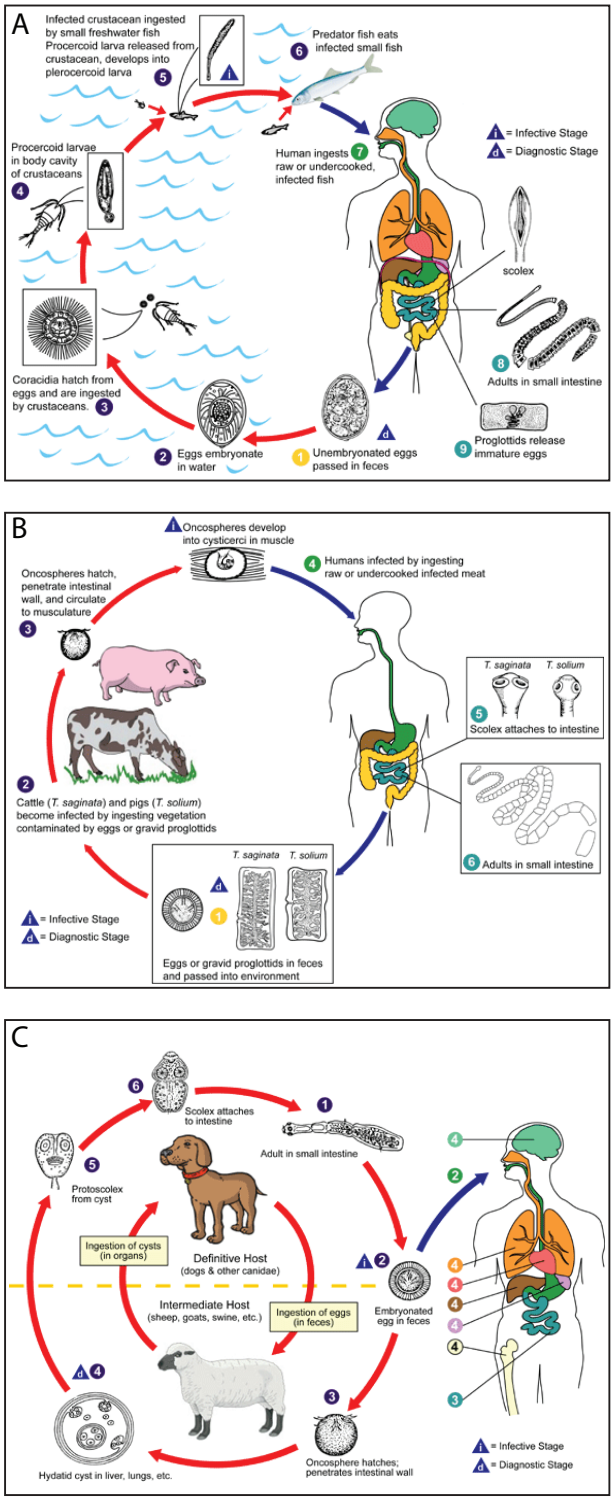

Figure 1.1 Examples of tapeworm lifecycles. The lifecycles of tapeworms are often complex, involving multiple hosts and employing different strategies. A) The broad fish tapeworm, *Diphyllobothrium latum*, B) the beef tapeworm, *Taenia saginata* and pork tapeworm, *T. solium*, C) the dog tapeworm, *Echinococcus granulosus*. Lifecycle images modified from Centers for Disease Control and Prevention, 2017.

1.2 Adaptations to parasitism

Tapeworm morphologies are highly reduced, lacking digestive, respiratory and circulatory organ systems. Instead, absorption of nutrients and excretion of waste products (although tapeworms do also possess an excretory system that includes flame cells and osmoregulatory canals) occurs across the tegument, which is covered with microtriches. Reductions in body plans, coupled with numerous adaptations ensure that tapeworms are highly specialised to an endoparasitic lifestyle. Some of these adaptations are described below, including those that aid in tapeworm attachment, protection and proliferation.

All tapeworm embryos develop into a hexacanth larva that is armed with six hooks. Eucestode (or 'true tapeworm') larvae are called oncospheres and are highly adapted to infect the intermediate host. Oncospheres have a highly-developed muscle system that coordinates movements of the hooks (Swiderski, 1983; Hartenstein and Jones, 2003). Coupled with enzymatic secretions from penetration glands, the oncosphere uses the hooks to burrow through the gut wall of the intermediate host. The oncosphere then undergoes a metamorphosis into the metacestode. It is thought that this is a true metamorphosis with most differentiated cells discarded whilst germinative cells proliferate into adult features, such as the scolex (Rybicka, 1967; Freeman, 1973; Świderski, 2007). Metacestodes vary highly in morphology with individual species often having specialisations specific to the infection of an intermediate or final host (Freeman, 1973; Chervy, 2002;

Świderski, 2007). The cyst body is a derived feature of cyclophyllidean metacestodes that acts as an adaptation for protection whilst in the intermediate host. During early development of the metacestode, a cavity (the primary lacuna) forms (Fig. 1.2). As the metacestode continues to develop, the tissue surrounding the cavity develops into a cyst wall into which the developing juvenile worm is encysted (Fig. 1.2). As such, the juvenile worm is protected and the metacestode lies dormant until ingested by the final host.

As previously described, in fully infective tapeworm metacestodes the juvenile worm is surrounded by a protective cyst. Once the metacestode infects the definitive host, the juvenile worm excysts and discards this cyst tissue. The juvenile worm possesses a fully developed scolex (Fig. 1.2 & 1.3 A-B) that allows the worm to attach to the mucosal wall of the host intestine. The scolex is a muscular, highly adapted attachment organ that can be armed with grooves, outgrowths, tentacles, suckers, hooks or a proboscis-like rostellum (Smyth and McManus, 1989). These attachment structures are all extremely well innervated, with nerves that connect to the simple ‘brain’ of the tapeworm – referred to as the cephalic ganglia (Halton and Maule, 2004). This ‘brain’ is comprised of lateral ganglia that are connected by a transverse commissure (Fig. 1.4). Two lateral nerve cords (LNCs) and four median nerve cords (MNCs) extend posteriorly from the cephalic ganglia and anterior extensions run up to the suckers and rostellum (Fig. 1.4).

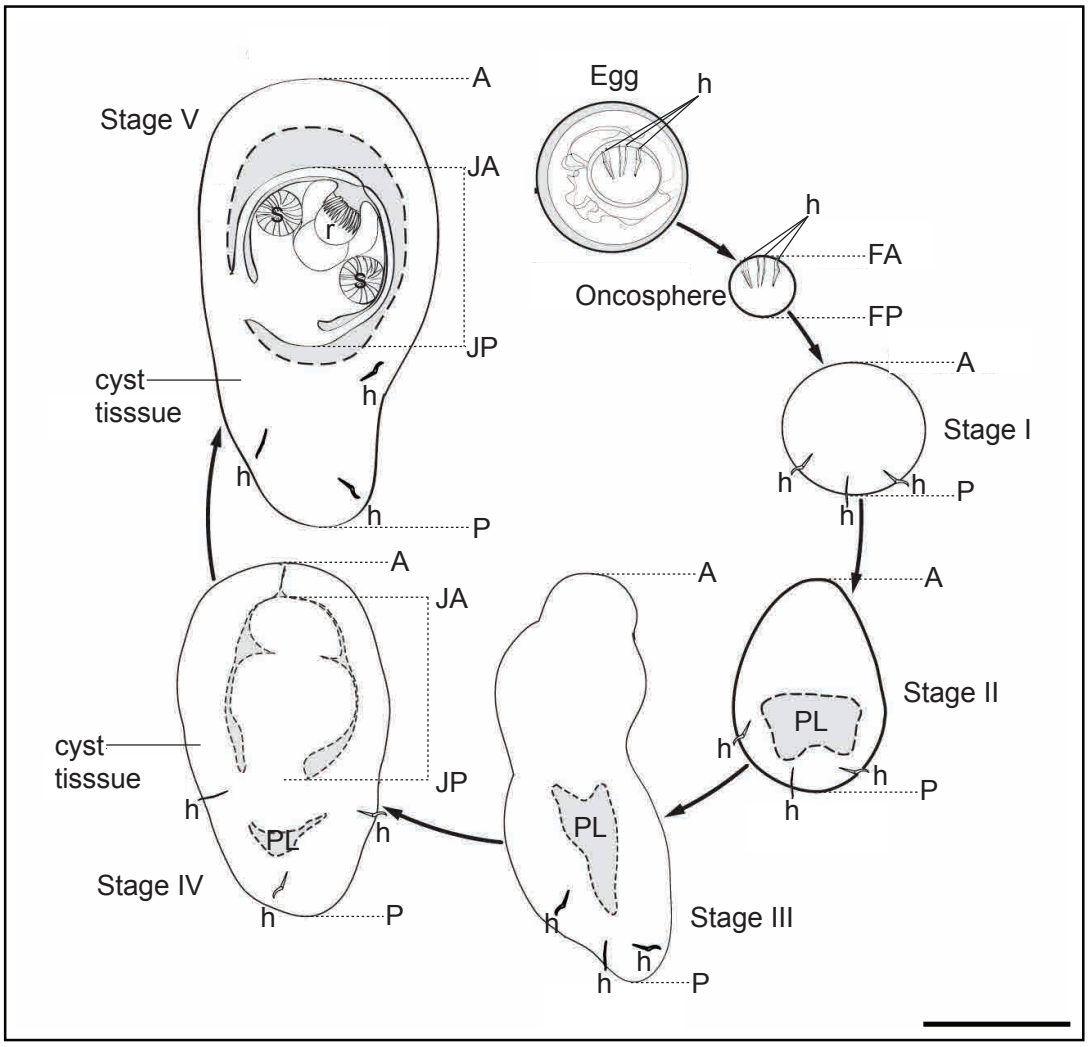

Figure 1.2 Development of *Hymenolepis microstoma* larvae. Eggs are released in the faeces of mice and are ingested by suitable intermediate hosts e.g. the flour beetle, *Tribolium confusum*. The oncospheres have three pairs of hooks at the functional anterior and uses them to burrow into the haemocoel of *T. confusum*. Development is staged into five phases, according to Voge, (1964). Between Stage I and II, a posterior cavity (the primary lacuna) develops and the larvae grows and becomes pear-shaped. At Stage III, the larva has elongated further and begins to narrow. The period between Stage III and IV occurs extremely quickly. The anterior of the stage III larva is drawn into the primary lacuna and is surrounded by the posterior tissues. The anterior goes on to develop into the juvenile worm whilst the posterior becomes the protective cyst tissue. At this point, the larva has two anteroposterior poles – that of the juvenile worm and also the entire larva. The juvenile worm develops further, encased by the cyst, until features including the suckers and rostellum are visible. Once the larva has developed to this stage it is fully infective and is referred to as a Stage V larva or the metacestode. *H. microstoma* metacestodes are also called cysticercoids A = anterior pole, FA = functional anterior, FP = functional

posterior, h = hook, JA = juvenile anterior, JP = juvenile posterior, PL = primary lacuna. Bar = 100 μm

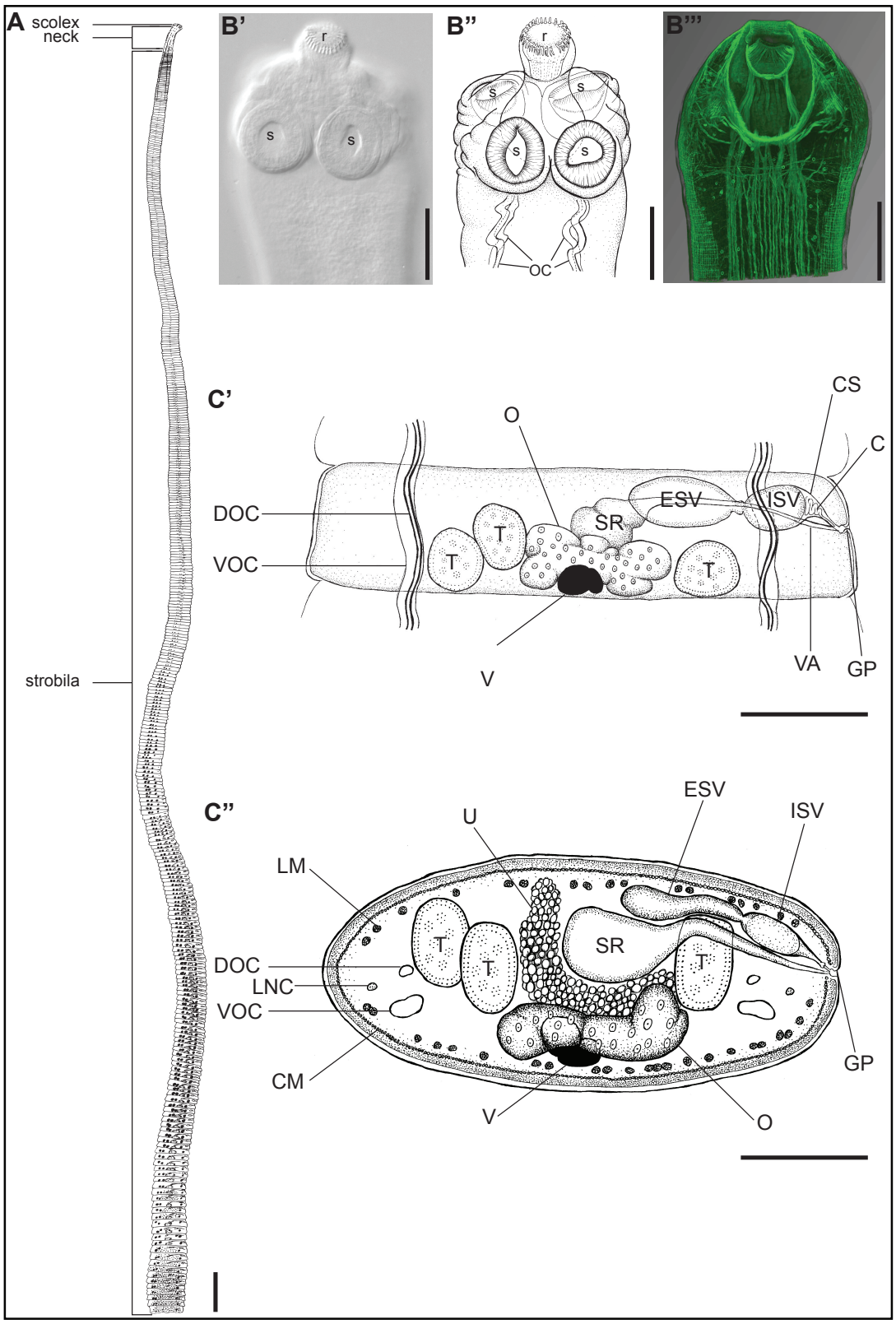

Figure 1.3 The anatomy of adult *Hymenolepis microstoma*. A) Illustration of the adult, which can be sub-divided into three regions – the scolex, neck and strobila. B') Light microscopy image of the scolex, B") illustration of the scolex and B"') musculature of the scolex after staining with phalloidin (in green). The scolex is highly adapted, armed with muscularised attachment

organs that allow the adult worm to attach itself to the small intestine and bile duct of its definitive host, the mouse. Illustration of a mature segment viewed C') ventrally and C") in cross section. DOC = dorsal osmoregulatory canal, ESV = external seminal vesicle, GP = genital pore, ISV = internal seminal vesicle, LM = longitudinal muscles, LNC = longitudinal nerve cord, O = ovary, OC = osmoregulatory canals, r = rostellum, s = suckers, SR = seminal receptacle, U = uterus, VOC = ventral osmoregulatory canal, VG = vitelline gland. Bars: A = 1mm, B = 10 μm, C = 100 μm. (Adapted from Cunningham and Olson, 2010 and A. Gruhl, OlsonLab, unpublished)

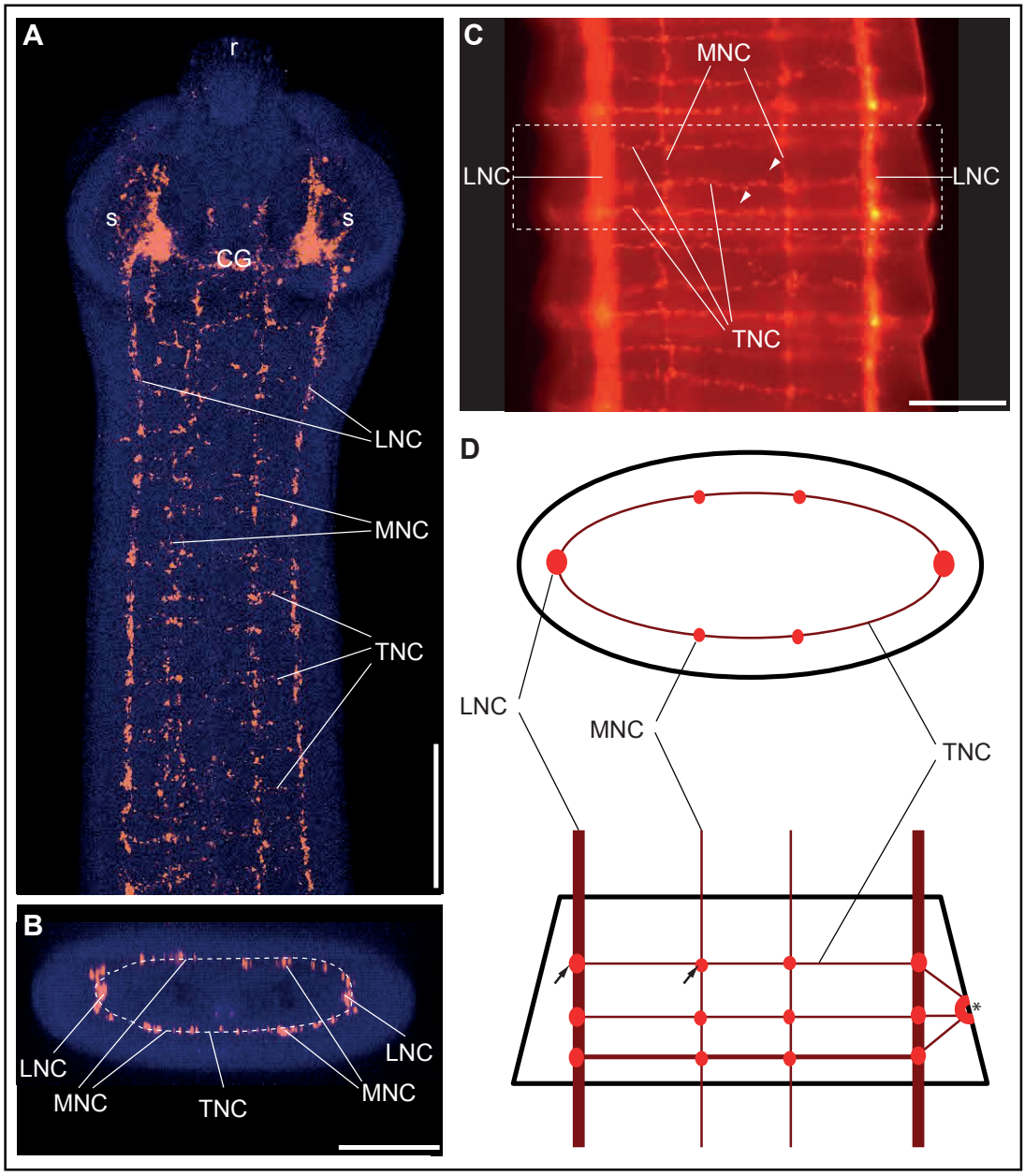

Figure 1.4 The nervous system of *Hymenolepis microstoma*. The nervous system is visualised by the neuronal marker anti-synapsin (red) alongside staining of nuclei by DAPI (blue). A) The general architecture of nerves in the scolex and early neck and B) late neck in cross section. C) Staining against synapsin (red) in mature segments. Box indicates one segment, arrowheads highlight minor nerves. D) Cartoon showing the location of the nerve cords in cross section and dorsally in one segment. Two longitudinal nerve cords run the length of the strobila, along with four median nerve cords. These are connected by three transverse nerve cords within each segment, arrows point to the junctions between these nerves. The asterisk indicates the genital pore, that is surrounded by a ring of nerves. CG = cephalic ganglia, LNC = lateral nerve cord, MNC = median nerve cord, r = rostellum, s = sucker, TNC = transverse nerve cord. Bars: 100 μm

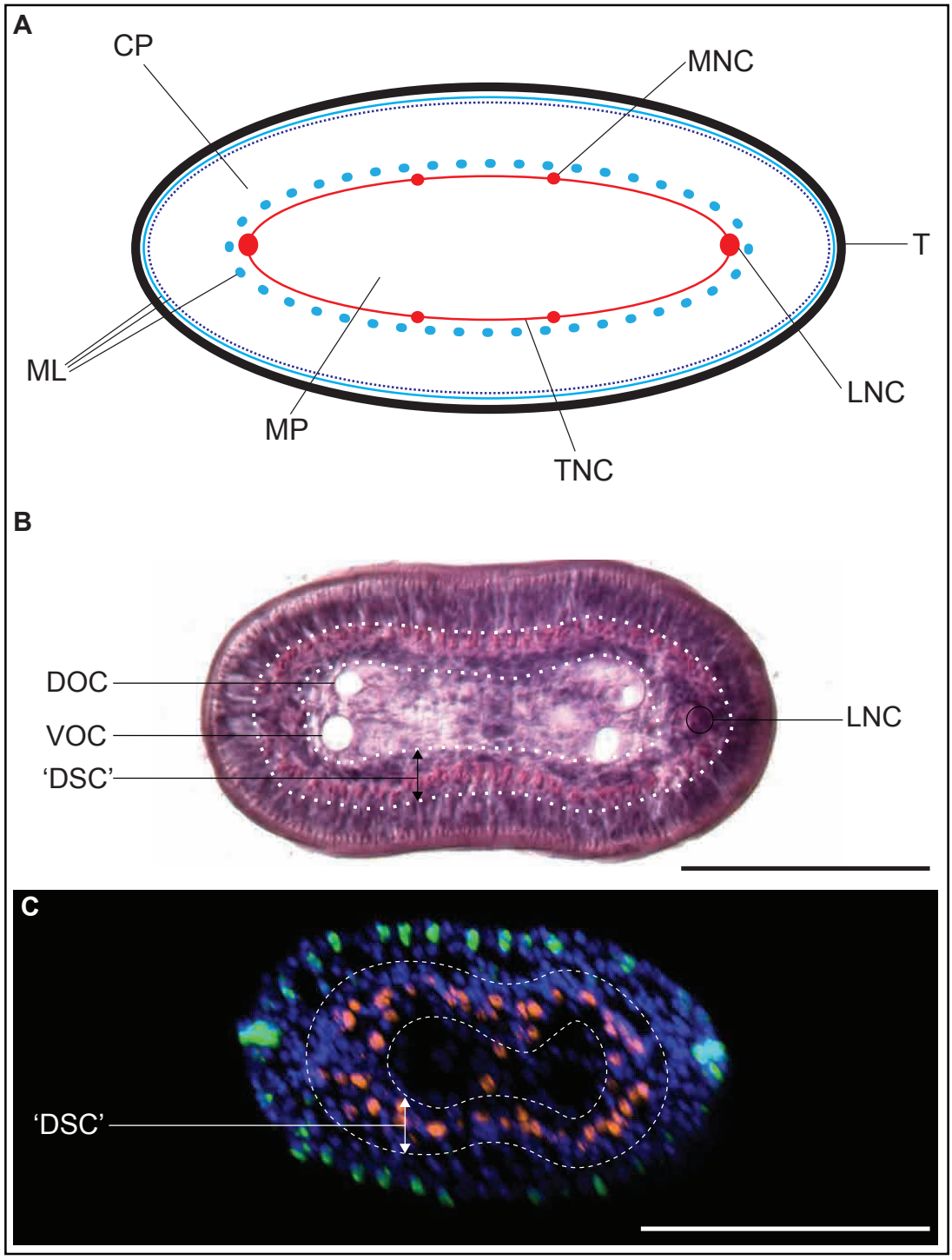

Figure 1.5 The developmental 'signalling cylinder' of *Hymenolepis microstoma*. The developmental 'signalling cylinder' (DSC) contains muscle, germinative cells and nervous tissue all in a close spatial association. A) Cartoon of the location of nerves and muscle layers in the neck in cross section. B) Histological cross section of the neck. The longitudinal nerve cords run through the middle of the DSC, as do all other major nerves. The ventral and dorsal osmoregulatory canals sit inside the DSC. C) Cross section of the neck stained with EdU (red), showing the location of germinative cells alongside staining of muscle with *Hmic-Collagen* (green)

and nuclei by DAPI (blue). DOC = dorsal osmoregulatory canal, DSC = ‘developmental signalling cylinder, LNC = lateral nerve cord, VOC = ventral osmoregulatory canal. Bars: 100 μm. (B) Adapted from OlsonLab, unpublished and C) adapted from A. Baillie/ OlsonLab, unpublished)

The juvenile body is initially mostly just scolex with an extremely short body. As the juvenile worm grows, the body begins to extend, forming the neck (Fig. 1.3 A), a region rich in germinative cells (Koziol et al., 2010) (Fig. 1.5). In the medullary region of the neck the genital primordia are initially observed as a 'streak' through the centre of the worm. Proglottisation refers to the serial repetition of gonads along the tapeworm body within the medullary tissue (Fig. 1.5). As proglottisation is a series of repeated units, it can be considered akin to segmentation. Indeed, one definition of segmentation is the serial repetition of units or body parts.

Proglottisation is continuous and each repeated unit of male and female gonads is called a proglottide. Towards the end of the neck, muscles in the cortical tissue (Fig. 1.5) begin to pinch and 'package' each proglottide into an externally divided unit, that cestodologists term a segment (Fig. 1.3 C-D). This external division of segments in tapeworms is a form of strobilation.

A chain of segments joined end-to-end forms the main body, or strobila (Fig. 1.3 A) of the adult worm and may be categorised into three stages of development – immature, mature and gravid. Immature segments are found closest to the neck and once proglottides are fully developed are considered to be mature (Fig. 1.3 C). The posterior-most segments are effectively uteruses that are full of eggs. In these segments, the reproductive organs have senesced, eggs are fully developed with viable infective embryos and are referred to as being gravid.

The processes of proglottisation and strobilation, although coupled in most tapeworms, appear to have evolved separately as an adaptation to an endoparasitic lifestyle. Proglottisation allows for an enormous increase in reproductive output. Indeed, the radiation of the Cestoda suggests an evolutionary trend towards increased fecundity (Hoberg et al., 1997; Olson et al., 2001). This increase is most apparent in the broad fish tapeworm, *Diphyllobothrium latum*, which is estimated to produce 1,000,000 eggs per day (von Bonsdorff, 1977). The benefit of strobilation is less clear, but one thought is that it reduces competition to the adult worm by allowing juvenile worms and eggs to develop and grow in a new environment niche.

1.3 Interrelationships and evolution of cestodes

There is a huge amount of diversity within the cestodes, both in terms of their complex lifecycles and development strategies. Phylogenies based on molecular data and morphological characteristics both show the Cestoda to be a monophyletic group (Dunn et al., 2014; Laumer et al., 2015). Early opinions suggested that all tapeworms were strobilate but that the condition was secondarily lost (Hyman, 1951). Advances in molecular phylogenies have since proved this not to be the case, and that basal taxa are not proglottisised and non-strobilate with an evolutionary drive within the cestodes to the derived, proglottisised and strobilate condition observed in higher cestodes (Fig. 1.6) (Hoberg et al., 1997; Olson et al., 2001). Whilst the general overview of tapeworm evolution is accepted, the monophyly of some groups still needs to be resolved (Olson et al., 2012), as do the

interrelationships of some clades (e.g. the “typanorynchs”) (Hoberg et al., 1997; Mariaux and Olson, 2001; Olson et al., 2001). However, the current understanding of broader cestode phylogenies is highly informative in guiding our understanding of the evolution of different developmental tactics (Figs. 1.6-7).

The Cestodaria represent the most basal clades within Cestoda (Fig. 1.7) and are comprised of the Gyrocotylidea and Amphilinidea. Adults of these two clades are monozoic, possessing a single set of reproductive organs that lack a common aperture, and have a simple attachment organ, or holdfast, (rather than a true scolex) (Xylander, 2001). The lycophore larvae of Gyrocotylidea and Amphilinidea swim using a ciliated epidermis. The lycophore is passively ingested and once in the gut of a suitable intermediate host, it actively infects the host, penetrating the intestinal wall with ten hooks coupled with enzymatic secretions from penetration glands (Rohde, 1994).

The remaining lineages of the cestodes are the Eucestoda or ‘true tapeworms’ (Figs. 1.6-7). The larval stage of these tapeworms is the oncosphere. Caryophylilideans have oncospheral larvae but adults only possess a single set of reproductive organs (displaying no proglottisation) (Fig. 1.6). This morphology, coupled with molecular data, suggests that caryophyllideans represent the base of the eucestodes (Hoberg et al., 2001; Olson et al., 2001; Waeschenbach et al., 2007) (Fig. 1.7).

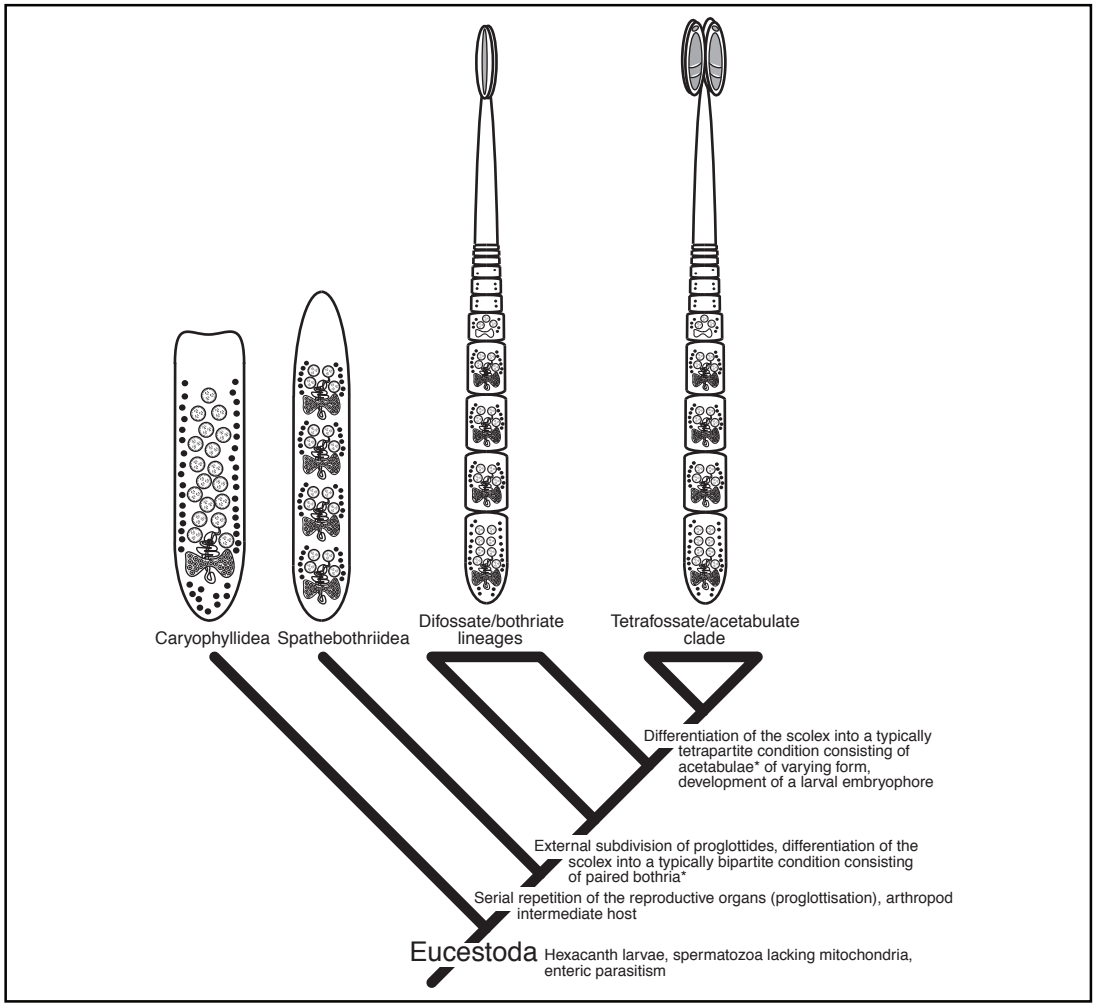

Figure 1.6 The stepwise evolution of body plans within the Eucestoda. The stepwise evolutionary pattern of tapeworm body plans that results in the proglottisised, strobilate condition observed in higher eucestodes. Basal eucestodes display neither of these characteristics. (From Olson et al., 2001).

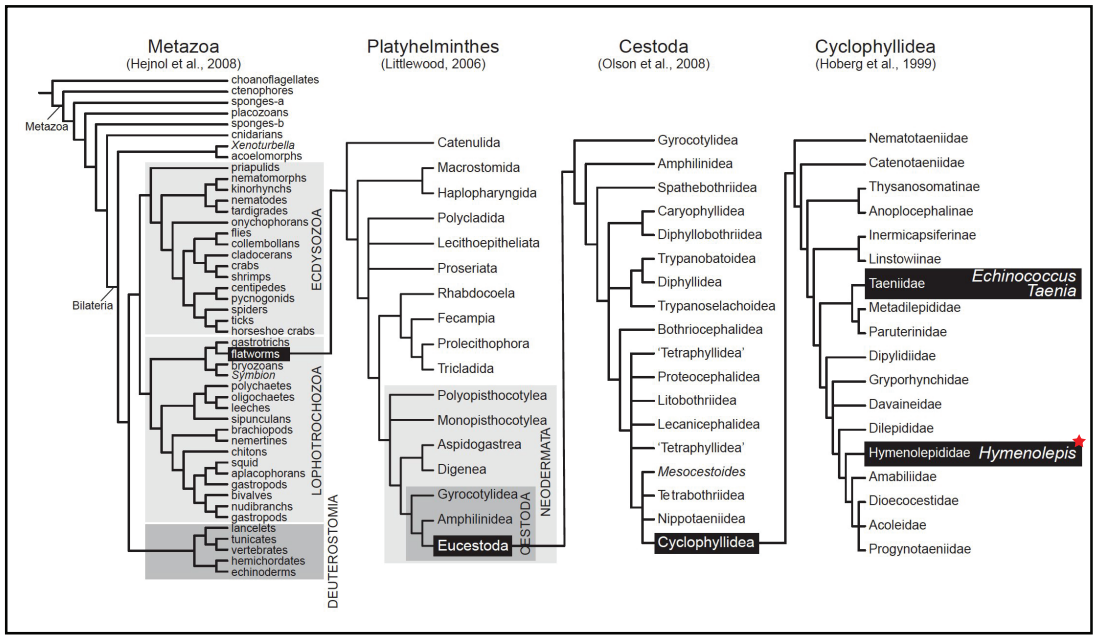

Figure 1.7 Phylogenetic position of the Cestoda. The position of cestodes amongst Platyhelminthes and more broadly within the Metazoa. Star indicates the position of Eucestoda within Cestoda and the position of the mouse bile duct tapeworm, *Hymenolepis microstoma* (Hymenolepididae). (Adapted from Olson et al., 2012).

The remainder of the eucestodes represent the bulk of all tapeworm species and here the evolutionary drive towards polyzooy begins (Figs. 1.6-7). The Spathebothriidea display the first steps of this drive towards the 'typical' tapeworm body plan (Olson et al., 2001). They are proglottisised but not strobilate, and have a simple scolex lacking true bothria or suckers. The general body plan of the Spathebothriidea clearly indicates the clade represents the intermediate step of tapeworm body plan evolution (Fig. 1.6).

The more derived eucestodes possess a strobila that is proglottisised and externally subdivided i.e. strobilated (Fig. 1.6). Based on the differentiation of the scolex, strobilate tapeworms are grouped into two further clades – the difossates and tetrafossates. The tetrafossates represent the most derived and species rich group and include the cyclophyliideans (Fig. 1.6-7). The Cyclophyliidea (Fig. 1.7) contains some of the most important species in terms of their impact on human health as agents of NTDs i.e. *Taenia* spp and *Echinococcus* spp. Given that strobilation is absent from the most basal groups and all other flatworms, it can clearly be interpreted as a derived condition.

Our understanding of tapeworm evolution shows that both proglottisation and strobilation are derived characteristics that have evolved independently. Proglottisation evolved as a means with which to increase fecundity – the serial repetition of proglottides allows for the production of an enormous number of progeny (Stunkard, 1962). Strobilation is a derived condition (Olson and Caira, 1999; Olson et al., 2001, 2008), although its benefits are

less obvious. The most likely explanation is that it allows eggs and juveniles to develop and grow in a new niche, thus reducing competition to the parent worm. Despite understanding the step-wise evolution of proglottisation and strobilation in tapeworms (Fig. 1.6), we still understand very little about the actual process of these conditions either morphologically or molecularly.

1.4 Exceptions to the rules

Proglottisation and strobilation are clearly derived conditions in the organisation of the tapeworm body plan. As is often the case, however, there are always a few exceptions to the rules. These deviants include 1) dioecious species within the Dicocestidae and Dicotaenia. 2) A secondary loss of strobilation, which can be observed in *Anantrum tortum* (Olson et al., 2001). 3) The secondary delay of proglottisation. This is observed in the little-known *Haplobothrium globuliforme*. Adults employ paratomy, in which the worm splits in a plane perpendicular to its AP axis, effectively joining worms end-to-end. The body plan of *H. globuliforme* is complicated further by the presence of two adult forms: the primary and secondary worm. Juveniles develop into adults that process a primary (or true) scolex and a primary strobila that bears several developing pseudoscoleces (MacKinnon and Burt, 1985a). The primary strobila does not develop sexually, instead proglottides develop behind the pseudoscoleces. The pseudoscoleces eventually bud off, becoming secondary scoleces that reattach further down the gut wall of the host, growing secondary strobila, with sexually developed proglottides (MacKinnon and Burt 1985a; MacKinnon and Burt 1985b), that is effectively

an entirely discrete worm. This secondary delay in the onset of proglottisation is unique within cestodes.

1.5 Barriers to research within Cestodology

The study of tapeworms provides numerous challenges to researchers and their unique development makes comparisons with other animals notoriously challenging. Difficulties even arise when considering the histology and cytology of tapeworms. The structure of the syncytium hinders histological interpretations with muscle, nervous and connective tissue becoming hard to disentangle after sectioning and staining (Young, 1935; Jones, 1945; Douglas, 1961; Lumsden et al., 1980). As internal parasites, the development of tapeworm *in vitro* culture methods is vital to uncovering more about their biology. However, tapeworms are not overly amenable to *in vitro* culture. The systems currently in place are exceedingly complex, often only resulting in the maintenance of worms for several days (Pouchkina-Stantcheva et al., 2013). Difficulties in developing *in vitro* culture techniques in tapeworms that are standard practice in free living flatworms (Newmark and Sánchez Alvarado, 2002) imposes a barrier to our understanding of tapeworm biology and development.

As well as difficulties in the techniques available to cestodologists, debates within the research community centring on the ontogeny, orientation, polarity and individuality of tapeworms have hindered our understanding of tapeworm development. These debates and conflicting views have resulted in a poor

understanding of tapeworm biology. This poor understanding of tapeworm morphology and development makes direct comparisons with other animals difficult, thus hindering our understanding of evolutionary novelties that are unique to cestodes. These debates have been on-going for over one hundred years (Young, 1935; Stunkard, 1962; Minelli, 2003; Olson, 2008) and although many have become conventionally accepted, they are still to be confirmed.

1.5.1 Historical questions surrounding tapeworm individuality

The argument as to whether the adult tapeworm is an individual or a colony has been around for many years (Young, 1935; Stunkard, 1962). Arguments favouring the 'colonial interpretation' centred on the idea that segments are individual, discrete units joined end-to-end, that resemble a budding linear colony. Wardle and McLeod (1952) described the strobila as a chain of individuals with a "tendency to remain attached to each other and to the parent body". Occasionally, inconsistencies in the dorsoventral (DV) polarity of proglottides within segments occur. For example, in the mouse bile duct tapeworm, *Hymenolepis microstoma,* segments possess three testes, two are aporal whilst the other is poral (Fig. 1.3 C). However, this pattern can sometimes be reversed (Cunningham and Olson, 2010). This is likely to occur through developmental errors. However, the DV inconsistencies within some tapeworm segments in general lead to postulate that segments represented individuals within a colony (reviewed in Stunkard, 1962). Young (1935) was also of the opinion that tapeworms employing proglottisation

resembled a colony, more comparable with the strobila of *Aurelia* spp jellyfish. In his opinion, as an individual hydroid could be solitary, or part of a colony within the jellyfish strobila, the tapeworm strobila should also be considered as a colony.

Arguments centring on the idea that the adult worm should instead be interpreted as an individual, pointed out that whilst sexual organs are serially repeated throughout the worm, all other organ systems display 'manifest organic unity' and that repetition can be interpreted as a unique phenomenon (Stunkard, 1962). Segments are continuous, with shared elements of musculature, nervous and osmoregulatory systems across the length of the strobila (Hyman, 1951). There are no internal boundaries between segments (Mehlhorn et al., 1981); indeed, staining of muscles with phalloidin has shown that the distinction between segments to merely be a "pinching" of circular muscles (OlsonLab, unpublished). The inconsistencies that are sometimes observed in the DV axes of individual segments are likely to occur through developmental errors. Furthermore, there are no specializations within individual segments and those that are damaged cannot be replaced or regrown. Finally, larval stages are individuals and therefore, the adult forms that they develop into should be considered as an individual too. The evidence supports the argument that the adult tapeworm is an individual rather than a colony, and the community now generally accepts this.

1.5.2 Heads or tails? Questions regarding anteroposterior polarity

It is generally accepted that the scolex of the worm is anterior, but this has only recently been confirmed using molecular techniques (Chapter 5; Koziol et al., 2016). This work looked at the expression patterns of genes belonging to the Wnt pathway (*Sfrp* and *Sfl*) that are well-known anterior markers in planarians and other metazoans (Gurley et al., 2008; Petersen and Reddien, 2008; Petersen and Reddien, 2009a, 2009b; Almuedo-Castillo et al., 2012). Expression of these two genes was found within the scolex of metacestodes and adult worms, confirming the scolex as anterior. Prior to this, the subject of tapeworm AP polarity had been a matter of fierce debate, largely focussing on comparative morphology (Young, 1935; Wardle and McLeod, 1952; Stunkard, 1962; Minelli, 2003, 2016; Olson, 2008; Egger, 2016).

Tapeworms do not have an obvious ‘head’. The scolex is highly reduced, possessing a simple ‘brain’ (the cephalic ganglia) that consists of a concentration of anteriorised nervous tissue. This generally appears as two lateral ganglia joined by a ring-like transverse commissure with neural projections and nerve plexuses extending into the attachment organs (Halton and Maule, 2004) (Fig. 1.4). Other than the suckers (and associated nerves), the scolex lacks any other sensory organs and this has resulted in a reluctance by some to identify the scolex as the anterior of the tapeworm (reviewed in Stunkard, 1962). Instead, some believed the scolex to be homologous with the posterior attachment sucker of trematodes (reviewed in Stunkard, 1962). The sentiment that the density of nervous tissue within the

scolex is only a necessity for controlling suckers (and other attachment organs) through innervation of muscle is echoed more recently (Halton and Maule, 2004). Halton and Maule (2004) do not argue that the scolex is not anterior. Instead they suggest that the development of muscles into attachment organs (such as suckers), the arrival of bilateral symmetry and the need to organise sensory information emanating from attachment organs imposed selection pressures on the nervous systems of tapeworms (and other flatworms). It is likely that these selection pressures rather than cephalisation drove the evolution of flatworm brains (Halton and Maule, 2004). Furthermore, the highly reduced nature of tapeworm morphologies in general and the, relative, simplicity of the tapeworm nervous system should be placed within the context of their parasitic lifestyles (Halton and Maule, 2004). The scolex should not be considered posterior; rather it is an anterior organ coupled with an overall reduced sensory system in tapeworms that has developed as an adaptation to endoparasitism.

The anterior nature of the scolex and overall polarity of tapeworms is further confounded when considering larval development. The oncospheral larvae of the more derived eucestodes have a set of six hooks at one pole and no brain. The hook-bearing pole is often referred to as the ‘functional anterior’ as the hooks are actively used to penetrate host tissue. During metamorphosis from oncosphere to metacestode, the scolex develops from the pole opposite that bearing the hooks. This has led some to believe that a total reversal of the AP axis occurs between larval and adult stages (reviewed in Stunkard, 1962; Freeman, 1973; Olson, 2008). For others, the

development of the scolex from what was considered to be the ‘posterior’ of the larvae led to the interpretation that the scolex is posterior and the strobila anterior, likening the attachment organs of the scolex to the posterior suckers of trematodes (reviewed in Stunkard, 1962).

Consideration of the lycophore larvae of more basal tapeworm clades and comparative analysis of the positioning of the hooks in both lycophores and oncospheres helps resolve the debate (Koziol, 2016). Lycophores have ten hooks that the larvae use to swim with. The pole opposing that bearing the hooks has a brain comprised of ganglia with tightly packed neuropil (Rohde, 1994), which higher eucestode larvae lack. As such, they have a clear AP axis and the hook-bearing pole is considered posterior. When in the gut of a potential host, the lycophore bends the hook-bearing pole into contact with the host and uses them to penetrate the host tissue. It is not too hard to imagine a scenario in which the oncosphere was derived from a lycophore through extreme reduction of the nervous system (Koziol, 2016). Hooks would have been retained as a means with which to penetrate the gut wall of the intermediate host. Indeed, the ‘intermediate’ step can even be observed in the ciliated larvae (coracidia) of some eucestodes that swim using their hooks and point them in the opposite direction to that in which they are travelling in (Freeman, 1973).

Finally, adult tapeworms travel in the direction of the scolex. Amongst the bilateria, the pole in which the direction of travel occurs is generally accepted as anterior. In most animals, this is more easily accepted due to the

presence of an obvious ‘head’ region. As such, the scolex of tapeworms should also be considered to be anterior.

The evidence outlined above points to the scolex as the anterior of the adult, and no inversion of AP polarity during development from larval to adult stages. Final confirmation is required to put an end to this debate. With the advancement of new molecular techniques (outlined later in this chapter), these questions can finally be laid to rest (Chapter 5; Koziol et al., 2016).

1.6 Unanswered questions regarding tapeworm development.

As alluded to previously, there are several questions pertaining to the development of tapeworms. Firstly, is the scolex anterior? Does a reversal of polarity occur between larval and adult stages? What are the genetic factors guiding the formation of the AP axis in tapeworms? Finally, what are the genetic factors controlling strobilation in tapeworms and are these factors modified in species with unusual body plans?

A handful of developmental signalling pathways control embryonic development across the Metazoa, namely the Wnt, Notch, Hedgehog (Hh), TGF-β and Receptor Tyrosine Kinase pathways (Gerhart, 1999; Pires-daSilva and Sommer, 2003). Notch and Hh signalling are implicated in segmentation and somitogenesis whilst Wnt is involved in the patterning of the AP axis (Gerhart, 1999; Couso, 2009; Petersen and Reddien, 2009b; Ingham et al., 2011). Given their role in other metazoans, these three

signalling pathways represent strong candidates with which to investigate tapeworm AP polarity and strobilation.

1.7 What is segmentation?

Defined by Henderson's Dictionary of Biology (2005) as 'series of essentially similar segments along the length of the body', the term segmentation often proves controversial and is mired in semantic debate. Clear definitions are lacking and are often generic and vague, misleading or even result in a 'conceptual trap' (Minelli & Fusco, 2004). A particular problem associated with the word 'segmentation' is that it describes both a process and a morphological feature (Minelli and Fusco, 2004). The process generally follows a molecular subdivision within tissues, where transcription factors are expressed along gradients or with an initial polarity. This leads on to the formation of segment borders that result in the morphological appearance of segments. Finally, the AP polarity of individual segments are established. Molecular studies have vastly improved our understanding of segmentation in individual species. However, the process is not restricted to the actions of a small handful of genes. Instead, the molecular processes involved are highly complex and so varied that an increasing wealth of molecular data has hindered in the creation of a precise definition of segmentation (Hannibal and Patel, 2013).

Traditionally, only arthropods, annelids and vertebrates were considered to be 'truly' segmented (Davis and Patel, 1999). However, recently, some

authors have urged a relaxation or redefinition of segmentation (Minelli, 2017). Budd (2001) suggested that the evolution of segmentation resulted from a successive accrual of repeated organ structures. He argued that segmentation should not be considered to be a trait of an entire organism, rather it is an attribute of an organ system. I.e. any repeated organ structure or system should be considered to be segmented. Following this interpretation, Hannibal and Patel (2013) suggest that 'segmentation' be used in a more general sense, coupled with a description of the specific segmented system in question. Their definition relates to the repetition of units, each with their own AP polarity, along the AP axis of the body (Hannibal and Patel, 2013). Subsequently, Graham et al. (2014) have also embraced a more generalised view of segmentation. First, in part, as there is no one single process driving segmentation – studies have uncovered a great number of genes that are involved in segmentation (Peel et al., 2005) with the precise role of homologous genes and gene families during segmentation often interchanging between different animals (reviewed in Hannibal and Patel, 2013). Second, that segmentation itself is nothing more than a morphological description of non-homologous structures (Graham et al., 2014).

Different tissue types (ectoderm, mesoderm and endoderm) can be segmented. However, in arthropods and annelids, segments are derived from both ectoderm and mesoderm. Vertebrate segments are derived from mesoderm, but segmental patterns are observed in other tissue layers, contributing to the overall patterning of each entire segment (Seaver, 2003;

Hannibal and Patel, 2013). If segmentation is a reiteration of structures along the AP axis, then (although controversial) a single cell within a column of cells or a single tissue layer could be considered to be segmented (reviewed in Hannibal and Patel, 2013). For a single cell to be deemed a segment, it must have a discernible AP axis (with molecular and or morphological asymmetry) (Hannibal and Patel, 2013). This is perhaps most convincingly observed in the notochord of the tunicate *Ciona savignyi* that is comprised of a single column of cells (Jiang et al., 2005). In the *C. savignyi* notochord, genes belonging to the planar cell polarity pathway, *Prickle* and *Strabismus*, are polarised within each cell. They are both anteriorised as the cells elongate, whilst in wildtype individuals, nuclei are found posteriorly (Jiang et al., 2005). This molecular and morphological asymmetry within each cell has led to the interpretation by some that single cells can be considered to be individual segments (Hannibal and Patel, 2013).

Repeated structures and units exist in many animals traditionally not considered to be ‘segmented’. As such, current definitions of the term should be updated. Following a more encompassing view of segmentation, descriptions of individual taxa should not be referred to as ‘segmented’, ‘non-segmented’ or ‘pseudo-segmented’. This terminology describing whether or not an animal is segmented often becomes confusing and redundant. Although a contentious issue, authors are now beginning to suggest that debates to determine if an animal is segmented or not should be discarded (Minelli and Fusco, 2004; Graham et al., 2014; Minelli, 2017). Instead, the segmental nature of specific structures and features should be discussed.

Therefore, segmentation is observed in animals traditionally considered to be ‘non-segmented’ including nematodes, molluscs, rotifers and tapeworms (Minelli and Fusco, 2004; Minelli, 2017).

1.8 Mechanisms of segmentation

Serial repetition of organs and whole structures along the AP axis is a common theme throughout the Bilateria. Body plans comprised of coordinated repeating units (or segments) are clearly observed in arthropods and annelids but are also observed in the metameric patterning of the skeleton, muscles and nervous system of vertebrates. Segmentation is found within three major clades – Deuterostomes, Ecdysozoa and Lophotrochozoa. The evolutionary origins of so-called ‘true segmentation’ are unclear (Seaver, 2003; Blair, 2008; Chipman, 2010). More recently, Graham et al. (2014) suggested that there is no one segmentation process, and that it likely evolved in vertebrates at least three times using different mechanisms. Despite the push to view segmentation in the context of an organ system or unit rather than an individual animal, the processes of ‘true segmentation’, i.e. animals that have traditionally been described as segmented (arthropods, vertebrates and annelids) remain best understood. A short overview of segmentation in these clades is described below.

1.8.1 Vertebrate segmentation

In vertebrate segmentation, or somitogenesis, somites (paired blocks of mesoderm on either side of the notochord) arise sequentially from the anterior of the elongating pre-somatic mesoderm (PSM) (Aulehla and Herrmann, 2004). Somitogenesis acts through a clock/wavefront mechanism that relies on direct cell-to-cell signalling and is often referred to as the oscillation or segmentation clock (Palmeirim et al., 1997; Pourquié, 2003). A series of coordinated oscillations of gene expression occurs in the posterior of the PSM that then move in a wave towards the anterior of the PSM, giving rise to the expression of 'segmentation genes' that eventually lead to the formation of somites in the anterior of the PSM. The clock/wavefront requires the complex interaction of three signalling pathways: Notch, Wnt and Fibroblast Growth Factor (Pourquié, 2003; Aulehla and Herrmann, 2004; Goldbeter and Pourquié, 2008; Ozbudak and Pourquié, 2008; Gibb et al., 2010) that are involved in the segmentation of all vertebrate species studied so far.

1.8.2 Arthropod segmentation

In *Drosophila melanogaster*, a cascade of several different suites of developmental genes is responsible for segmentation including maternal effect genes, gap genes, pair-rule genes and finally the segment polarity genes *Wingless/Wnt* (*Wnt*) and *Engrailed* (*En*) (reviewed in Hartenstein and Chipman, 2015). The mechanisms acting in *D. melanogaster*, however, are

extremely derived and do not represent the segmentation process of most arthropods (Peel et al., 2005). Unfortunately, despite this, *D. melanogaster* remains the best understood system of segmentation. However, a common theme uniting the segmentation of many arthropods is the requirement for Wnt signalling to control overall AP polarity of segments. Hedgehog signalling has also been implicated in conferring parasegmental boundaries in arthropods, whilst Notch signalling too is involved in the establishment of segments in some arthropods. However, this is not a universal role and in some species it functions to pattern segments post their morphological appearance (Lioa and Oates, 2017).

1.8.3 Lophotrochozoan segmentation

The annelids are a clear example of a group of animals displaying overt segmentation. Unfortunately, our understanding of segmentation within annelids is limited, restricted to a handful of taxa, often with conflicting results. Most studies have utilized candidate gene approaches based on arthropod and vertebrate systems (Bleidorn et al., 2015) and have uncovered some remarkable similarities between the molecular mechanisms of segmentation in annelids and arthropods.

The role of *En* as a universal 'segmentation gene' in annelids is a dubious one. In *Platynereis dumerilii* it seems to play a similar role to that observed in *D. melanogaster* (Prud'homme et al., 2003). However, this is not the case in

the leech, *Helobdella robusta*, or the polychaete annelids, *Capitella* sp. I and *H. elegans* (Seaver and Shankland, 2001; Seaver and Kaneshige, 2006).

Wnts seem to play a role in annelid segmentation and are generally expressed in stripes within the ectoderm (Cho et al., 2010; Janssen et al., 2010). In some cases though, *Wnts* are thought to maintain segment patterns rather than establish them (Dray et al., 2010). Hedgehog signalling, too, is implicated in the maintenance of segments rather than their establishment (Dray et al., 2010). The role of Notch signalling in the segmentation process of annelids seems much clearer with *Notch* expression occurring in an oscillatory manner (Song et al., 2004; Rivera et al., 2005) resembling the clock/wavefront mechanism observed in vertebrates. Further evidence in a role for Notch signalling in annelid segmentation is observed after inhibition and knockdown of notch factors that result in segmentation defects (Rivera and Weisblat, 2009).

1.9 Mechanisms of strobilation in Cnidaria

Other than tapeworms, the only other animals to display strobilation are the sycphozoan and cubozoan jellyfish. Unfortunately, very little is understood about the development of these animals as they have proved to be highly difficult to culture in laboratory conditions (Technau et al., 2015). However, we do know that Wnt signalling patterns the body axis of other cnidarians. Several *Wnts* are expressed in rings around the oral pole of *Nematostella vectensis* (Kusserow et al., 2005; Lee et al., 2006) as is *β-catenin*

(Wikramanayake et al., 2003) whilst the *Wnt* antagonist *Sfrp* is expressed at the aboral pole (Kumburegama et al., 2011). In *Hydra*, *Wnt3* is expressed in the oral organiser (Hobmayer et al., 2000). These patterns suggest that Wnt signalling is crucial in establishing oral-aboral polarity in cnidarians. More recent work also suggests that Hh signalling also plays an important role in patterning the oral-aboral axis of *N. vectensis* (Matus et al., 2008), again echoing our understanding of Wnt and Hh signalling acting as posterior organisers. Finally, Notch signalling is required for patterning and division of regions along the oral-aboral axis, where it maintains the border between tentacles and the head (Münder et al., 2013), linking Notch, Wnt and Hh with patterning of the cnidarian body plan.

1.10 Should tapeworms be considered as segmented?

As described previously, a precise description of segmentation can be difficult to define, and should be given within the context of the particular segmented unit or structure (Budd, 2001). Here, segmentation refers simply to the repetition of units along the AP axis of the animal. In the context of tapeworms, proglottisation and strobilation can both be interpreted as forms of segmentation. In the case of proglottisation, the repetition of proglottides (units of male and female gonads) along the length of the adult tapeworm is clearly a type of segmentation. Strobilation is a form of reproduction. However, the resultant morphology of the process (i.e. the external packaging of proglottides into segments) and their repetition along the AP

axis of the strobila means that strobilation can also be interpreted as segmentation.

Whilst there is a superficial resemblance of tapeworm strobilation with the segmentation observed in other phyla, these two processes are not likely to be homologous (Seaver, 2003; Blair, 2008). However, a role for Notch, Wnt and Hh signalling in the patterning of segments in vertebrates and invertebrates and in establishing polarity in non-strobilate cnidarians suggests that they could be controlling strobilation in tapeworms. By investigating these pathways during tapeworm strobilation, we can piece together how segmentation arose *de novo* in this group and whether the same so-called 'segmentation' genes observed in other phyla have been co-opted to control tapeworm strobilation.

1.11 The planarian as a model of flatworm developmental biology

Planarians are highly plastic and have an incredible ability to regenerate and regrow missing organs after wounding. As long-lived animals, they are also able to replace aging differentiated cells lost during tissue turnover and can grow or shrink depending on the availability of nutrients. Recent advances in functional genomics have renewed interest in planarians, establishing them as popular models which to study development and regeneration (Newmark and Sánchez Alvarado, 2002).

1.11.1 The flatworm ‘neoblast’ as the source of all development

The presence of embryonic-like cells that are distributed across the entire body of many flatworms has long been observed (reviewed in Reddien and Sánchez Alvarado, 2004). These cells are extremely small, between 8-15 μm, and are round or slightly pear shaped (Baguñà and Romero, 1981). Based on their size and morphology, they represent ~25-30% of the total population of planarian cells (Baguñà and Romero, 1981; Baguñà et al., 1989). Referred to as neoblasts, they are highly undifferentiated with a large, prominent nucleus and very little cytoplasm (Reddien and Sánchez Alvarado, 2004). Neoblasts are the only dividing cells in planarians (Baguñà, 1976) and can be incorporated with an analogue of thymidine: 5-bromo-2’deoxyuridine (BrdU), that serves as a marker for proliferating cells (Newmark and Sánchez Alvarado, 2000). Neoblasts are required during planarian regeneration and homeostasis, with their progeny producing all known cell types (reviewed in Reddien and Sánchez Alvarado, 2004). This suggested that neoblasts could be totipotent stem cells. Pluripotent cells are essential during embryonic development, but are not present in adult tissues which instead are maintained by tissue-specific adult stem cells (Blanpain et al., 2004; Ohlstein and Spradling, 2006; Barker et al., 2007). As such, the heterogeneity of the neoblast population was uncertain and may have instead been comprised of multiple, indistinguishable, lineage-restricted cells (Reddien and Sánchez Alvarado, 2004).

Influential research by Wagner et al. (2011) finally solved the nature of planarian neoblasts. Through the development of several assays, they determined them to be pluripotent stem cells that are capable of self-renewal. It is these neoblasts that are responsible for the incredible regenerative powers of planarians. All dividing planarian cells express the *Piwi* gene, *Smedwi-1* (Guo et al., 2006). Irradiation eliminates these cells, but seven days post irradiation, clusters of *Smedwi-1*$^{+}$ cells are visible (Wagner et al., 2011). Assays incorporating BrdU resulted in *Smedwi-1*$^{+}$ and BrdU^{+} cells, indicating clonal growth rather than dedifferentiation and no other source of proliferating cells (Wagner et al., 2011). To test the potential of neoblasts, neuronal and intestinal markers were developed (as these are derived from different germ layers) and neoblast colonies were triple-labelled with these markers and a *Smedwi-1* antibody. Individual colonies contained cells of both lineages, indicating multipotency, and were distributed across the body. Singly transplanted neoblasts are able to recover lethally irradiated worms, highlighting their pluripotency (Wagner et al., 2011).

The morphological heterogeneity of neoblasts and the expression of *Piwi* by all neoblasts indicates a homogenous population of cells. However, further investigations into the potential of individual neoblasts has challenged this (reviewed in Zhu and Pearson, 2016). Only a subset of injected neoblasts (so-called clonogenic neoblasts) were able to rescue lethally irradiated planarians, suggesting functional heterogeneity (Wagner et al., 2011). Single cell transcriptomics have uncovered evidence for three sub-populations of neoblasts (van Wolfswinkel et al., 2014; Wurtzel et al., 2015). A fourth

subclass has since been identified (Currie et al., 2016). Functional data is still lacking for these sub-populations of neoblasts and it is still uncertain if some neoblasts are truly totipotent, whilst others are dedicated specialists to one tissue type (Zhu and Pearson, 2016).

1.11.2 Signalling and transcription factors patterning the planarian AP body axis

Animal AP axes are specified during early embryonic growth and canonical Wnt signalling has been uncovered as a posterior organiser that helps to establish and pattern the AP axis across the Metazoa (Petersen and Reddien, 2009b). This is a common feature in all bilaterians and is considered an ancestral state (Holland, 2002; Martin and Kimelman, 2009; Niehrs, 2010). *Wnts* are expressed in a "polarised manner along the primary axis" of pre-bilaterians (Petersen and Reddien, 2009b) including the sponge, *Amphimedon queenslandica* (Adamska et al., 2007), the starlet sea anemone, *N. vectensis* (Kusserow et al., 2005; Lee et al., 2006) and *Hydra* (Hobmayer et al., 2000). Conversely, *Wnt* inhibitors, including *Sfrp* are expressed anteriorly. In vertebrates, disruption of Wnt signalling leads to severe axial defects during development (Liu et al., 1999; Kiecker and Niehrs, 2001).

Wnt signalling also patterns the AP axis of planarians. Several *Wnts* are expressed in the posterior of *Schmidtea mediterranea*: *Wnt1*, *Wnt11-1* and *Wnt11-2* are restricted to the tail whilst *Wnt11-5* is observed along a gradient

in a posteriorised manner during homeostasis (Petersen and Reddien, 2008, 2009a; Adell et al., 2009; Gurley et al., 2010). Conversely, inhibitors of the pathway (i.e. *Sfrp-1* and *Sfrp-2*) are expressed in the anterior of the planarian (Gurley et al., 2008; Petersen and Reddien, 2008; Gurley et al., 2010). Inhibition of *β-catenin* via RNA interference (RNAi) leads to severe AP defects from "radial-like hypercephalisation" to "tailless" planarians (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). This evidence strongly suggests that canonical, *β-catenin* driven Wnt signalling is required for the maintenance of AP polarity, with the inhibition of *β-catenin* driving anterior growth. Similarly, *Wnts* are key to re-establishing the AP axis after injury. Knock down of *β-catenin* during regeneration results in head formation at posterior facing wounds (i.e. two-headed worms), as does *Wnt1* RNAi (Kobayashi et al., 2007; Adell et al., 2009; Petersen and Reddien, 2009a; Gurley et al., 2010; Almuedo-Castillo et al., 2011). Inhibition of antagonists, such as *APC* (a *β-catenin* inhibitor), results in growth of a new tail at the anterior pole (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). Wnt signalling is also required during planarian embryogenesis. During the very first stages of embryogenesis, *Wnt1* and *Sfrp* are co-expressed, resembling patterns observed during adult regeneration. By the time the worms have become juveniles, both genes have become polarised, echoing the expression patterns of intact adults during homeostasis (Martín-Durán et al., 2010; Martín-Durán and Romero, 2011). These results highlight the conservation of canonical Wnt signalling in the specification of the AP axis across the Metazoa.

It is not only Wnt signalling that plays an important role in planarian AP patterning. Hedgehog signalling is also required for the establishment and maintenance of the AP axis in both *S. mediterranea* and *Dugesia japonica* (Rink et al., 2009; Yazawa et al., 2009). Silencing of Hedgehog signalling factors via RNAi during regeneration results in “no-tail” (and very rarely two-headed) individuals and combined knockdowns lead to greater AP defects. Knockdown of *Patched* (the *Hh* receptor) generates two-tailed animals. *Hh* was also found to act upstream of Wnt signalling, and therefore modulating *Wnt* activity and expression (Rink et al., 2009). A model has also been postulated that indicates *Hh* signals to be transported posteriorly along axons that then activate Wnt signalling (Yazawa et al., 2009).

1.11.3 Muscles as the source of ‘positional control genes’ in planarians

Many of the genes involved in planarian axial patterning can be described as ‘position control genes’ (PCGs), that are responsible for instructing regeneration and tissue turnover (Witchley et al., 2013). Witchley et al. (2013) define planarian PCGs as genes that are a) regionally expressed along a body axis (or axes) and b) either result in an abnormal expression patterns after RNAi or are predicted to regulate signalling pathways. However, the identities of cells providing positional information during regeneration remained elusive. Transplantation experiments (Saló and Baguñà, 1985; Kato et al., 2001; Witchley et al., 2013) suggested that the source of positional information required during regeneration (and homeostasis) are differentiated cells.

The expression of many PCGs share a common feature in that they are expressed in a subepidermal cell layer (Reddien et al., 2007; Petersen and Reddien, 2009a, 2011; Gaviño and Reddien, 2011), a region devoid of neoblasts (Witchley et al., 2013). Unlike neoblasts, irradiation does not stop expression of PCGs (Reddien et al., 2007; Petersen and Reddien, 2009a, 2011; Gurley et al., 2010; Gaviño and Reddien, 2011), indicating that PCGs are expressed in non-neoblast cells (Witchley et al., 2013). The subepidermal expression of PCGs suggests that a population of cells in this location is responsible for providing positional information during homeostasis and regeneration. This population of cells are termed 'positional control cells' (PCCs) (Witchley et al., 2013). Assays performed by Witchley et al. (2013) confirmed that PCGs are all expressed in the same cells and that these are muscle cells (determined by co-expression with the muscle markers troponin and collagen). Most of the muscle cells expressing PCGs are subepidermal, however, a small population of intestinal and pharangeal muscle cells also express PCGs (Witchley et al., 2013). Muscle is therefore a 'major site of instructive signalling', providing positional instructions for planarian (Witchley et al., 2013).

1.12 The planarian as a blueprint for parasitic flatworm development

Over the last ten years or so, there has been an incredible resurgence in planarian research. The recent 'comeback' of planarians is largely due to advances in genomic resources, culturing techniques and development of molecular markers (Newmark and Sánchez Alvarado, 2002). As such, the

development of these techniques has resulted in a planarian 'toolkit' that, coupled with the incredible plasticity and regeneration powers of planarians has led to them becoming popular research models. This 'renaissance' in planarian biology is rapidly evolving, furthering knowledge on their biology including stem cell, cell turnover, axial patterning and cell signalling in general. By 'borrowing' from the planarian 'toolkit' we can begin to vastly improve our understanding of parasitic flatworms.

1.13 Evolutionary developmental biology of parasitic flatworms

There has been a historical lack of developmental biology within parasitic animals generally – most research tends to focus on classical model systems, such as *D. melanogaster*, mouse, and more recently, planarians. Commonplace tools available to researchers of free-living flatworms (e.g. specific cell markers, *in vitro* culturing systems and RNAi) are lacking in tapeworm research. However, we can use the recent surge of research in planarians to guide and further research methodologies in tapeworms. In other words, borrow from the planarian 'toolkit'. Indeed, several advances into classical and emerging parasitic flatworm models are taking place and this has largely been due to the progress taking place in planarian systems. Some of the major advancements in three tapeworm models are described below.

1.13.1 *Echinococcus multilocularis*

The availability of the *E. multilocularis* genome (Tsai et al., 2013) and the development of comprehensive *in vitro* culture systems has established the species as a laboratory model for the development of taeniid tapeworms (Smyth et al., 1966; Brehm and Spiliotis, 2008). *In vitro* protocols have developed from so-called first generation (i.e. co-culturing) techniques to second generation methods. In first generation systems, *E. multilocularis* tissue is co-cultured with host cells. Three methods exist; the first, tissue-block, uses blocks of metacestode tissue that is cultured in serum containing host feeder cells (Hemphill and Gottstein, 1995). The second, developed by Jura et al. (1996) embedded host hepatocytes on a layer of collagen, to which homogenised *E. multilocularis* tissue was added and supplemented with serum. The third, large-scale liquid cultivation, incubated homogenised metacestode tissue in liquid culture alongside rat hepatocyte feeder cells that were added weekly to the culture rather than grown alongside parasite tissue (Spiliotis and Brehm, 2009). These first generation methods, although successful, did not represent the true situation of metacestode growth within a host *in vivo*. The second generation system does not co-culture with host feeder cells (Spiliotis et al., 2004). *E. multilocularis* vesicles are initially grown under the conditions described by Jura et al. (1996) and are then filtered to remove host tissue (Spiliotis et al., 2004). Reducing agents are added to the media under hypoxic conditions, allowing vesicles to grow. This second generation method of culturing *E. multilocularis in vitro* provides a long-term, axenic system that successfully replicates complete development from

germinative cells into metacestodes *in vivo* (Spiliotis et al., 2004; Brehm and Spiliotis, 2008). These culturing methods have enabled transfection experiments (Brehm and Spiliotis, 2008; Spiliotis et al., 2008) and have produced a robust system with which to study germinative cells further (Koziol et al., 2014).

The development of successful RNAi methodologies is vital to understanding gene functionality. RNAi in *Echinococcus* spp have had various success rates (Mizukami et al., 2010; Hu et al., 2015). However, the success of the *E. multilocularis* culturing system has resulted in a reproducible and highly robust RNAi method in primary cell cultures (Spiliotis et al., 2010) which will surely aid in functional studies of metacestode development.

The differentiated somatic cells of flatworms do not proliferate. Instead, all flatworms have a population of undifferentiated stem cells that are the only cells to divide (reviewed in Reddien and Sánchez Alvarado, 2004). Planarian stem cells are referred to as neoblasts whilst cestode models have identified 'germinative cells' that are the functional equivalent of neoblasts. The ability to culture larval cells of *E. multilocularis* has helped uncover a unique stem cell system. The germinative cells of *E. multilocularis* (and other tapeworm species) have a similar morphology to planarian neoblasts – small, with few cytoplasmic extensions, they have a large nucleus with a prominent nucleolus and basophilic cytoplasm. *E. multilocularis* germinative cells in primary cell cultures are the only proliferating cells and are homogenous in their morphology (Koziol et al. 2014). Like planarians, there is heterogeneity at the molecular level indicating that functionally distinct sub-populations

exist (Koziol et al. 2014) however, there are also some marked differences between the stem cells of tapeworms and free-living flatworms. All neoblasts express *Piwi* (Guo et al., 2006). However, *Piwi* and *Vasa* (two crucial stem cell genes) have been lost in cestodes (Tsai et al., 2013; Skinner et al., 2014). Paralogs of *Piwi* are expressed in *E. multilocularis* metacestodes, but not in all germinative cells, and are also expressed in post-mitotic cells (Koziol et al. 2014). More recently, a terminal-repeat retrotransposon in miniature has been identified as a marker for *E. multilocularis* germinative cells, although this is not a universal marker of all *E. multilocularis* germinative cells (Koziol et al., 2015). The molecular heterogeneity of *E. multilocularis* germinative cells certainly suggests that different lineages exist (Koziol, 2016).

1.13.2 *Mesocestoides corti*

The localisation of germinative cells in the tapeworm *Mesocestoides corti* (used as a model for murine neurocysticercosis), has been described (Koziol et al., 2010). Proliferating cells of *M. corti* are found in the medullary parenchyma (in a ring), close to the inner layer of muscle, but not in the cortical parenchyma or sub-tegument. Cell renewal in these regions occurs instead by the migration of cells from the medullary parenchyma. In larvae and adults, proliferative cells are more abundant towards the anterior of the worms, in the scolex and neck, and decreases towards the posterior. During early segmentation, clusters of dividing cells are observed before any visible signs of segmentation (Koziol et al., 2010). As proglottides develop, a cluster

of proliferative cells is observed in the genital primordia, indicating that these cells proliferate *in situ* and do not migrate from other regions of the body. As development continues and proglottides mature, only cells in the testes primordial and the outer cells of the genital primordium proliferate (Koziol et al. 2010). Fewer proliferating cells are observed in the cortex after strobilation, which suggests that once segmentation has occurred, cell renewal of the cortex is less important and proliferative cells are directed to the development of gonads (Koziol et al., 2010).

1.13.3 *Hymenolepis diminuta*

Alongside functional genomic data, several atlases of tissue architecture have been produced in planarians (Zayas et al., 2010; Chong et al., 2011) and the blood fluke, *Schistosoma mansoni* (Collins et al., 2011). These use readily available reagents, including plant lectins, antibodies and other stains with broad cross-reactivity. Until recently, a tapeworm-specific atlas was lacking (Rozario and Newmark, 2015). These atlases are essential, providing the foundations to better understand (and complement) genomic studies. The atlas of the model tapeworm *H. diminuta* (Rozario and Newmark, 2015) provides a detailed map of the musculature, nervous and osmoregulatory systems and gonads utilising techniques developed in other flatworms and is vital to truly understand the anatomy of tapeworm signalling. For example, whilst longitudinal muscles and nerves run continuously along the length of the strobila, there are also regionalised transverse muscles and nerves in each segment (Rozario and Newmark, 2015).

Overall, the advances stemming from many flatworm models are forwarding our understanding of the phyla. Firstly, flatworm signalling is continuous with new cells stemming from pluripotent stem cells (neoblasts and germinative cells). Disparities amongst the stem cells of flatworms clearly exist (at both the molecular and morphological level), suggesting that sub-populations of stem cells are present throughout the phyla. In all flatworms, there is also a close spatial association of nerve, muscle and stem cells (Wikgren et al., 1971; Halton and Maule, 2004; Koziol et al., 2010; Rozario and Newmark, 2015) and cell signals clearly emanate from this group of different cell types. Finally, cestode signalling is not restricted to the neck region. Although stem cell proliferation is more abundant in the neck, it is not restricted to this region, with germinative cells distributed across the body (Koziol et al., 2010).

1.14 *Hymenolepis microstoma* as a model system for tapeworm developmental biology

1.14.1 *Hymenolepis microstoma*: an ideal laboratory model

The mouse bile duct tapeworm, *Hymenolepis microstoma*, (Figs. 1.2-3 & 1.8) is a cosmopolitan species (Dvorak et al., 1961) that infects mice. It is a typically strobilate worm, and thus is an ideal model system to investigate tapeworm AP polarity and strobilation in. Although less well studied than other members of the genus (i.e. *H. diminuta* and *H. nana*), *H. microstoma* has been used as a laboratory model since the 1950's (Stewart et al., 1975).

H. *microstoma* has several advantages over *H*. *diminuta* and *H*. *nana* as a laboratory model: it is smaller; (relatively) cheaper to maintain (as it infects mice rather than rats); is unable to auto-infect; is refractory to human infection and both its intermediate and final host are themselves model organisms – the flour beetle, *Tribolium confusum*, and the mouse, *Mus musculus*.

1.14.2 Lifecycle and biology

The lifecycle of *H*. *microstoma* (Fig. 1.8) is typical of many tapeworms in that it is complex, using two hosts. Adult worms are found in the bile duct of mice and release eggs that are passed out in the faeces. Eggs are ingested by an appropriate beetle host (e.g. *T*. *confusum*). During ingestion, the beetle mouthparts break the eggshells, releasing oncospheral larvae. Once inside the beetle gut, the oncospheres use three pairs of hooks and proteolytic secretions to burrow through into the haemocoel. In the haemocoel, the worms go through five stages of larval development (Voge, 1964) (Fig. 1.2) and undergo a complete metamorphosis, becoming infective cysticercoids within a week (Voge, 1964; Cunningham and Olson, 2010). Infected beetles are passively ingested by mice and factors found in the stomach (e.g. HCL) dissolve the larval cyst whilst factors in the duodenum activate the juvenile worms. Juveniles migrate into the bile duct where they attach themselves with four suckers and hooked rostellum (Fig. 1.3 B). Here, the juveniles begin to grow and strobilate. After approximately 16 days the tapeworms reach

maturity and are fully gravid (de Rycke and van Grembergen, 1966; Cunningham and Olson, 2010).

The nervous system and musculature of *H. microstoma* is typical of those found in other flatworms (Halton and Maule, 2004). Staining against synapsin allows visualisation of the nervous system (Fig. 1.4). Two longitudinal nerve cords (LNCs) and four median nerve cords (MNCs) extend from the highly innervated cephalic ganglia (Fig. 1.4) and are connected by transverse nerve cords (TNCs) in the neck and strobila (Fig. 1.4). Within each segment, there are three TNCs (Fig. 1.4 C-D), as is also observed in *H. diminuta* (Rozario and Newmark, 2015).

Anti-Phalloidin staining of the musculature of *H. microstoma* reveals a highly muscularised scolex, especially in the suckers and rostellum (Fig. 1.3 B, OlsonLab, unpublished). Longitudinal, transverse and circular muscles are, again, comparable to those of *H. diminuta* (Rozario and Newmark, 2015). Furthermore, *in situ* hybridisation of *Hmic-Collagen* (Fig. 1.5) identifies a sub-epidermal ring of myocytons (OlsonLab, unpublished) – the non-contractile part of the muscle cell containing the nuclei that is offset from the contractile myofibril (Halton and Maule, 2004; Witchley et al., 2013).
Edu staining indicates the localisation of proliferative cells to echo observations in *M. corti* (Koziol et al., 2010), with dividing cells observed in the medullary parenchyma (Fig. 1.5, OlsonLab, unpublished) and a greater abundance of these cells in the scolex and neck (OlsonLab, unpublished).

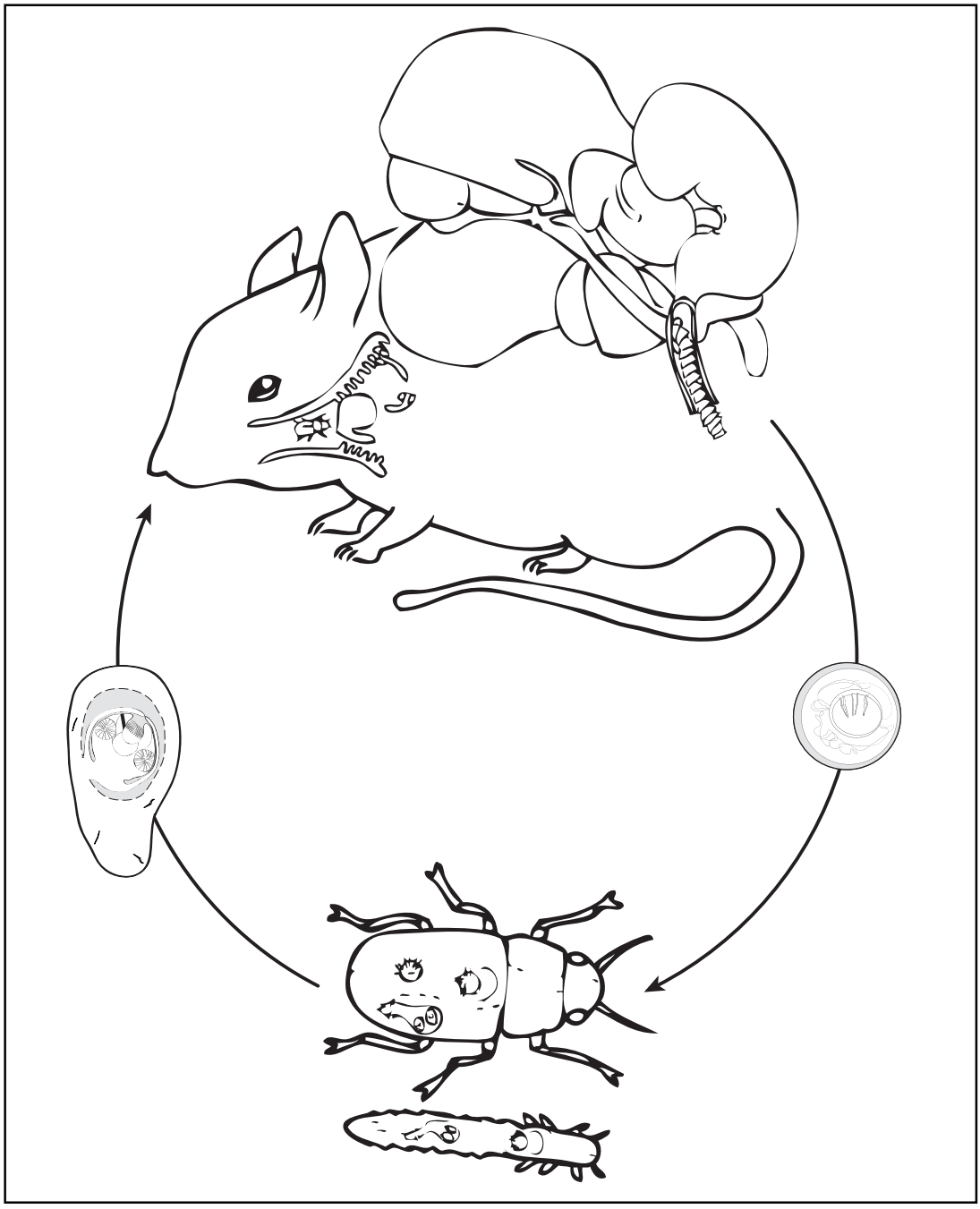

Figure 1.8 The life cycle of the mouse bile duct tapeworm *Hymenolepis microstoma*. Eggs are released in faeces and are ingested by a suitable beetle host. Here the eggs hatch into oncosphere and undergo development into an infective cysticercoid that is then ingested by a mouse. Once in the stomach, the juvenile worm activates and moves to the bile duct where it develops into and adult worm.

Traditional histology techniques and the use of molecular markers highlight the close spatial association of nerve, muscle and germinative cells that can be termed the 'developmental signalling cylinder' (DSC) (Fig. 1.5), as is found in other tapeworms (Wikgren et al., 1971; Halton and Maule, 2004; Koziol et al., 2010; Rozario and Newmark, 2015). Based on our understanding of planarian signalling, there is a strong indication that tapeworm muscle cells may be providing the positional information guiding germinative cells and that the nervous system could also be involved in the transportation of these signals.

1.15 The available *Hymenolepis* 'toolkit'

Developing techniques currently available in other flatworms, in *H. microstoma* will allow us to produce a *Hymenolepis* 'toolkit'. This toolkit is vital in furthering our understanding of tapeworm development.

i) Genome and gene models

The genome of *H. microstoma* has been published (Tsai et al., 2013). Analysis has shown tapeworm genomes to be small in comparison with those of free-living flatworms, and are highly reduced with losses of genes and pathways common in other animals (Olson et al., 2012; Tsai et al., 2013). The most notable losses are the within the *Hox* cluster and the complete loss of *ParaHox* genes (Olson et al., 2012; Tsai et al., 2013) alongside a huge reduction in the number of *Wnts* (Tsai et al., 2013). Around

a third of all *Wnt* genes have been lost in tapeworms (Riddiford and Olson, 2011). Surprisingly, tapeworms (and trematodes) have also lost the stem cell marker *Vasa* and *Piwi* sub-family that regulate germline stem cells. Instead, tapeworms have evolved a new sub-family of genes encoding argonaute proteins, suggesting the germinative cells of tapeworms are highly modified (Tsai et al., 2013). Other gene expansions have occurred in tapeworms including genes involved in the formation of the tegument, cell-cell adhesion and in a family of heat shock proteins (Tsai et al., 2013). The genome and accompanying gene models will allow identification of genes thought to be involved in the segmentation process and AP polarity in *H. microstoma*.

i) Transcriptomic profiling (RNA-Seq)

Alongside the genome, the transcriptome of *H. microstoma* has been characterised (unpublished data) from non-normalised cDNA of three biological replicates of 5-day-old larvae, whole adult worms and the neck/scolex, mid (i.e. mature) and end (i.e. gravid) regions from adult worms. This was performed using paired-end Illumina Hi-Seq data with 100bp read length and ~200bp insert size. Gene models have been produced with the most recent update totalling ~12500 genes (available at http://www.sanger.ac.uk/resources/downloads/helminths/hymenolepis-microstoma.html). The number of reads per kilobase of transcript per million mapped reads (RPKM) for each gene was calculated (Tsai et al., 2013) and can be used as a means of measuring (differential) gene expression.

ii) Colorimetric *in situ* hybridisation

Colorimetric whole mount *in situ* hybridisation (WMISH) using Digoxigenin (DIG)-labelled RNA probes (or riboprobes) has long been used as a means in which to visualise gene expression. WMISH techniques developed in planarians (Pearson et al., 2009; King and Newmark, 2013). WMISH techniques have been expanded into parasitic flatworm models, including trematodes (Cogswell et al., 2011) and have also been adapted to the *H. microstoma* model (http://www.olsonlab.com/) and other tapeworm models (Koziol et al., 2014).

iii) Fluorescent *in situ* hybridisation and tyramide signal amplification techniques

Colorimetric ISH methods generally use an antibody conjugated to alkaline phosphatase against a DIG modified UTP that relies on a reaction with a substrate (usually NBT/BCIP) to induce a colour change. Fluorescent *in situ* hybridisation (FISH) methods use a variety of hapten-modified UTPs (including DIG, fluorescein and biotin). Antibodies against these haptens are conjugated to horseradish peroxidase (HRP or POD). The tyramide signal amplification (TSA) substrate is then introduced and many molecules are deposited immediately around the primary antibody. In this way, the signal is amplified. The HRP activates the TSA substrate, causing it to form covalent bonds with tyrosine residues. The TSA substrate is conjugated to a fluorophore and can then be detected using fluorescence microscopy. HRP

activity can be inactivated or quenched with either H_2O_2 or sodium azide and allows for the detection of another transcript with a different fluorophore. Over the last five years or so, fluorescent ISH (FISH) systems have been developed thet allow double (dFISH and multi-FISH analyses in planarians (Pearson et al., 2009; King and Newmark, 2013). Modification of planarian FISH protocols can be used to further current methods in gene expression analysis in *H. microstoma*.

iv) *In vitro* culture and RNA interference

Maintenance of *H. microstoma in vivo* (in laboratory conditions) is relatively easy and simple. However, developing *in vitro* methods have been more troublesome. *In vitro* culture methods have been produced for *H. microstoma* from oncosphere to mature adult (Seidel, 1971; Seidel, 1975; Evans, 1980). However, recent attempts at replicating these techniques have proved unsuccessful (Pouchkina-Stantcheva et al., 2013). Functional data is key to understanding development and *in vitro* methodologies are vital in determining loss-of-function phenotypes. Pouchkina-Stantcheva et al. (2013) showed that RNA-mediated gene silencing, through RNAi, is functional in *H. microstoma* with expression of *post2* quantitatively suppressed by up to 80% by soaking juvenile worms with double-stranded RNA (dsRNA). The inability to culture adult worms *in vitro*, however, meant that loss-of-function phenotypes could not be determined. Two alternative methods were tested: 1) microinjecting larvae directly; 2) an *in vivo* approach whereby encysted larvae were soaked with dsRNA before inoculating them in mice.

Microinjecting was ultimately abandoned, whilst soaking was ineffective as dsRNA was unable to penetrate the cyst wall. Despite this, the capability of an effective RNAi mechanism that possibly acts via two routes (Pouchkina-Stantcheva et al., 2013) suggests the potential for using *H. microstoma* as a model for strobilation.

1.16 Thesis aims

Questions concerning strobilation and AP polarity remain unresolved in tapeworms. This thesis will provide the first insights into the developmental biology of the tapeworm, *H. microstoma*. By investigating developmental signalling pathways that coordinate segmentation and specify the AP axis in other metazoans (i.e. Wnt, Hh and Notch), I hope to uncover the genetic factors controlling tapeworm strobilation and AP polarity. Current paradigms in planarian research imply that free-living flatworms use both Wnt and Hedgehog signalling to specify posterior identity and control AP patterning. Outside the Platyhelminthes, Wnt, Hedgehog and Notch signalling systems have all been implicated in the coordination of AP patterning and segmentation. It therefore seems most prudent to begin investigations into tapeworm development by looking at these three systems in the model tapeworm *H. microstoma*. To do this, I will also develop some of the techniques used in free-living flatworms, including FISH methodologies. I will also undertake the development of a novel platform with which to investigate RNAi *in vivo*, borrowing from RNAi methodologies used in *Tribolium* spp

(Posnien et al., 2009). Lastly, I aim to compare the development of *H. microstoma* with tapeworms with atypical body plans.

The specific aims of this thesis are:

1. To investigate candidate developmental pathways controlling tapeworm polarity and segmentation. This will be done through characterisation and determination of the genomic components of the Wnt, Hedgehog and Notch pathways. The spatial expression patterns of these pathways will be characterised throughout the *H. microstoma* lifecycle.
2. To develop and expand tools for investigating gene expression and function in the model tapeworm *H. microstoma*. Namely, these will include FISH and TSA techniques allowing expression analyses and the development of a platform with which to investigate RNAi *in vivo*.

The work presented in this thesis will provide some of the first investigations into tapeworm gene expression patterns and will lead to the identification of genes relevant to AP patterning and strobilation in tapeworms.

Chapter 2

Materials and Methods

2.1 Maintenance of the *Hymenolepis microstoma* system in vivo

BALB/c mice infected with *Hymenolepis microstoma* were culled then dissected and adult worms were recovered from the bile duct. The gravid ends were removed and the tissue was macerated and spread thinly onto filter paper then fed to adult *Tribolium confusum* that had been starved for one week. After two days, the filter paper was removed and replaced with 1:1 wholemeal and white flour supplemented with dried yeast. After ten days, beetles were dissected in conditioned water and patent cysticercoids were removed. BALB/c mice were then infected with ~20 cysticercoids via oral gavage. Use of animals was approved and conducted in accordance with UK Home Office regulations under project license PPL70/7150 held by P.D. Olson.

2.2 Animal collection and fixation

2.2.1 Strobilar adults

Mice infected with *H. microstoma* for more than two weeks were culled. Adult worms were removed from the bile duct, anterior small intestine and stomach of mice and swirled in saline to remove debris. Individual tapeworms were transferred to near-boiling saline and swirled for ~5 sec to extend and kill before transferring to fresh 4% paraformaldehyde (PFA) in phosphate buffered saline + 1% Tween20 (PBST) (4% PFA/PBST) and left in the refrigerator overnight. Fixed worms were washed in PBST then cut into small

sections ~2-5 mm long and transferred to 1.5 ml microcentrifuge tubes in preparation for expression analyses. Specimens were either kept in PBST or dehydrated in a graded ethanol series and stored at 4 ^{o}C.

2.2.2 Pre-strobilar juvenile worms

Mice were infected then culled and dissected into supplemented culture media 24, 48, 72 and 96 hrs post-infection. Juvenile *H. microstoma* were removed from the upper third of the small intestine and fixed in fresh 4% PFA/PBST for 1 hr at room temperature (RT). Worms were transferred to 1.5 ml microcentrifuge tubes and washed in PBST at least four times, then stored in the refrigerator. The first signs of strobilar development were seen 96 hrs post-infection at which time most worms were in the region of the opening of the bile duct.

2.2.3 Larvae

A larval series was produced by carrying out timed collections of infected *T. confusum*. Adult *T. confusum* were starved for one week and then fed macerated gravid *H. microstoma* tissue spread finely on filter paper for two days. The filter paper was removed and replaced with flour, as above, 3 days post exposure to eggs. Beetles were dissected daily from 3-7 post-exposure. Larvae were collected, transferred to conditioned water to remove debris and then fixed in fresh 4% PFA/PBST and left in the refrigerator overnight.

Larvae were then washed in PBST and staged (from I - V) (Fig. 1.2) according to Voge (1964).

2.3 RNA extraction

Adult worms were harvested and ~30 mg of tissue from scolex/neck regions of several worms was collected and blotted to remove excess water. Tissue was homogenised in 600 µl RTL buffer (Qiagen) with β-mercaptoethanol. The lysate was centrifuged for 3 min at full speed and the supernatant transferred to a gDNA Eliminator spin column. RNA was extracted using the RNeasy Plus Mini kit (Qiagen). RNA was eluted in 30 µl RNase-free water, the concentration was quantified using a NanoDrop (Thermofisher) and the RNA stored at -80 ^{o}C.

2.4 cDNA synthesis

RNA samples were reverse transcribed into double stranded complimentary DNA using the SuperScript III First-Strand Synthesis System (Invitrogen) with oligo$(dT)_{20}$ primers. Samples were quantified using a NanoDrop and stored at -20 ^{o}C.

2.5 Gene identification and phylogenetic analyses

The published version of the *H. microstoma* genome (Tsai et al., 2013) and gene models (available at

http://www.sanger.ac.uk/resources/downloads/helminths/hymenolepis-microstoma.html) were imported into Geneious (Drummond et al. 2011) where they could be visualised and converted into a local database. Using Geneious, Notch and Hedgehog pathway orthologs were identified (Table 3.1 and 4.1) through reciprocal BLAST hits against the *H. microstoma* genome and gene models. Wnt pathway genes were previously identified (Riddiford & Olson, 2011), however, BLAST searches were performed to ensure no ortholog was missed. Genes were confirmed by screening against the NCBI non-redundant nucleotide database and their integrity checked by examining any conserved domains through NCBI's Conserved Domain Database (CDD) (Marchler-Bauer and Bryant, 2004; Marchler-Bauer et al., 2014) and EMBL-EBI's InterPro (Mitchell et al., 2014). Genomic and transcriptomic resources for *H. microstoma* include RNA-Seq data that provide the number of reads per kB of transcript per million reads mapped (RPKM). RNA-Seq data included larval samples (approximately half way through metamorphosis), whole adult worm samples, and three regions of the strobilar adults: the scolex and neck region, the mature strobila and the gravid strobila. RPKM data was used as a means with which to measure relative gene expression. Core pathway genes and those up-regulated in the neck/scolex or larvae were identified from RNA-Seq data and targeted for later WMISH analysis.

H. microstoma gene models were used as BLAST queries to identify orthologs in other Platyhelminthes. These were: 1) the taeniid tapeworms *Echinococcus multilocularis*, *E. granulosus* and *Taenia solium*; 2) the blood

flukes *Schistosoma mansoni* and *S. japonicum*; and 3) the model planarian, *Schmidtea mediterranea*. Where possible, searches were performed against the gene models rather than the genome data. Where genes were absent in *H. microstoma*, lophotrochozoan and *Drosophila* genes were used as query sequences. Occasionally, orthologs were curated so where possible we used published genes sequences and mined genomic resources such as GenBank and WormBase ParaSite (Howe et al., 2015; Howe et al., 2016). Multiple sequence alignments of *H. microstoma* genes, identified tapeworm and fluke transcripts and orthologs mined from genomic databases were performed in Geneious using MUSCLE. Alignments were visually inspected and edited. Gaps were removed to produce a final alignment that was used to produce a gene tree. Unrooted trees were produced using Bayesian inference based on the WAG amino acid substitution model. Resulting gene trees were used to confirm the relationships of genes with each other and within their groups.

2.6 Primer design

Gene specific primers for each gene of interest were designed against gene models using the Primer3 plugin in Geneious. Where possible, primers were designed to be between 18-22 bases long, have a GC content of 40-60%, melting temperatures within 3 ^{o}C of each other, and were unlikely to produce hairpins, self-dimers of primer-pair dimers. An ideal product length between 1800-2200 base pairs was aimed for, but depending on gene length and the previously described primer characteristics, this was reduced where needed.

Upon receipt, all primers were re-suspended in sterile water to a stock concentration of 100 µM from which a 10 µM working solution was prepared; both were stored at -20 ^{o}C. A complete list of gene specific primers and their corresponding gene models can be found in Appendix 1.

2.7 Amplification of genes of interest

Using gene specific primers (GSPs), gene transcripts (mRNAs) were amplified from larval or adult cDNA via PCR in a 25 µl reaction for 30 cycles. PCR products were loaded with GelRed and electrophoresed on a 1% agarose gel at 90 V for 45 min. The resulting gels were visualised using an UV illuminator. Correctly sized bands (based on expected product sizes) were either gel extracted using the QIAquick gel extraction kit (Qiagen) or purified with the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions.

2.8 Riboprobe synthesis

One µl of purified gene product was transformed, via TA cloning, into StrataClone SoloPack competent cells using the StrataClone Blunt PCR kit (Agilent) and blue-white screened for successful insert ligation. Half volumes of the recommended kit protocol were used, as these were found to be sufficient. An A-tailing protocol was used to increase ligation efficiency when gene products did not insert efficiently into the vector (see Appendix 2).

Successfully transformed colonies were selected and re-suspended into 500 µl ddH_2O and heated to 80 ^oC for 5 min to disrupt the cells and denature the plasmid DNA. Successfully inserted products were amplified via PCR using universal M13 primers. Amplified products were purified as above. Purified colony DNA was amplified via PCR (using the protocol described above, but using 25 cycles) with the universal M13 forward primer paired with forward and reverse GSPs to establish the directionality of the insert. Colony DNA was then amplified via PCR using both forward and reverse universal M13 primers in a 75 µl reaction to produce amplicons that included T7 and T3 promoter sites for reverse transcription of riboprobes. To confirm the identity of the cloned products, samples of purified colony DNA amplified with M13F/R primers were sent for Sanger sequencing at the NHM. The identities of the cloned products were verified using Geneious (Drummond et al., 2011).

Anti-sense RNA probes were synthesised from 1 µg of purified PCR product through RNA labelling with either digoxigenin-UTP (DIG) or fluorescein-12-UTP (FITC) by *in vitro* reverse transcription with either T7 or T3 RNA polymerase (dependant on insert directionality) using either DIG or FITC RNA Labelling Kit (Roche). Probes were precipitated in alcohol using lithium chloride. RNA pellets were re-suspended in 30 µl DEPC water, quantified on a NanoDrop and stored at -80 ^oC.

2.9 Whole-mount in situ hybridisation (WMISH)

In situ hybridisation is a method that uses labelled complementary (i.e. anti-sense) RNA probe that binds to mRNA and therefore allows the visualisation of gene expression. During this project, I have used and developed colorimetric WMISH and fluorescent techniques that use bench-made reagents and tyramide signal amplification (TSA). The protocols for each of these are described below.

2.9.1 Colorimetric WMISH

Worm sections were permeabilised with proteinase K at RT (adults for 10 min, larvae for 5 min) to remove outer proteins and allow better penetration of riboprobes. Worms were then rinsed in TEA and acetic anhydride (to aid with specific binding of the riboprobe) before washing in PBST. Specimens were re-fixed for 20 min in 4% PFA/PBST to maintain tissue integrity then washed thoroughly (at least five times) in PBST to remove all PFA. Worms were pre-hybridised in hybridisation (-) buffer (Hyb-) for 10 mins to equilibrate the specimens. Hyb- contains several reagents that aid hybridisation and reduce non-specific binding of the riboprobe. This includes formamide (which increases the stability of single stranded RNA), Tween20, CHAPs, Denhardt's solution and heparin that all act as a substrate and reduce non-specific binding and sodium saline citrate buffer (SSC) that controls the stringency of the hybridisation. Hyb- was removed and replaced with hybridisation (+) buffer (Hyb+) overnight at 60 ^{o}C. Hyb+ contains the addition

of yeast RNA that blocks non-specific hybridisation. Probes were diluted in fresh Hyb+ to a final concentration of 1 µg/ml and denatured at 80 °C for 3 min. Worms were left to hybridise in the probe solution overnight at 60 °C. The probe solution was removed from samples and kept at -20 °C for future use. Samples were rinsed twice with Hyb+ before washing in 2x SSC and 0.2x SSC at 60 °C which was followed by washing in maleic acid buffer (MAB) at RT. Worms were pre-incubated in MAB + 2% bovine serum albumin (BSA) + 20% heat-treated lamb serum (blocking buffer) for 2 hrs at RT on a rolling machine attached to a rocker at 15 rpm to prevent non-specific binding of the antibody. The solution was replaced with fresh blocking solution containing 1/2000 affinity purified sheep anti-digoxygenin antibody (Roche) coupled to AP. Tubes containing the worms were left overnight on a rocker at 4 °C. Antibody was removed and kept at 4 °C for future use. Specimens were washed thoroughly in MAB and then alkaline phosphatase buffer at RT. NBT/BCIP was added to the final wash of alkaline phosphatase buffer and worms were left in the dark until a chromogenic colour change developed. After staining, specimens were washed in PBST to stop the reaction and post-fixed in 4% PFA/PBST for an hour at RT. Worms were washed several times in PBST to remove the PFA and then put in 50% glycerol/PBST for at least 1 hr to clear. Specimens were then transferred into 80% glycerol/PBST for at least 1 hr to clear fully. Worms were stored in the refrigerator before mounting on glass slides in fresh 80% glycerol/PBST and stored at 4 °C. For a list of reagent recipes, see Appendix 3.

2.9.2 Fluorescent *in situ* hybridisation (FISH)

The initial steps for FISH follow the protocol above for colorimetric WMISH to the point of the addition of antibody. After blocking for 2 hrs, the blocking solution was replaced with fresh blocking buffer containing 1/50 affinity purified sheep anti-digoxigenin-POD (i.e. peroxidase) Fab fragments (Roche) and left overnight on a rocker at 4 ^{o}C. The antibody was removed and the worms washed once in PBST for 10 min and then twice in PBS + 0.1 M Imidazole (pH 7.6) for 10 min. All steps from this point on were carried out in the dark to protect the samples from light. Specimens were incubated in a bench-made fluorescein tyramide solution (Appendix 4). Adults were incubated for 10 min, larvae for 30 min. Specimens were washed at least four times in PBST and then cleared as described in the protocol for colorimetric WMISH.

2.9.3 Double FISH (dFISH)

During dFISH, two riboprobes are used to detect mRNA of two separate genes. The riboprobes for each gene are conjugated to two different haptens – DIG and FITC. Both probes can be hybridised simultaneously but need to be detected independently to prevent cross reactivity of the HRP. FITC-labelled riboprobes are less stable than those labelled with DIG. As such, the FITC-labelled riboprobe is detected first and the HRP enzyme used during the staining is quenched to stop cross-reactivity with the next riboprobe and

tyramide solution. The second, DIG-labelled riboprobe can then be detected using a different coloured tyramide.

The dFISH protocol first follows the methods described above for FISH, with the addition of a FITC-labelled riboprobe (in a concentration of 1 µg/ml) during probe hybridisation steps and the use of 1/50 affinity purified sheep Anti-Fluorescein-POD Fab fragments (Roche) during the first antibody incubation. After the fluorescein tyramide solution was removed and washed off, specimens were incubated in 100 mM sodium azide solution for 45 min to quench the HRP enzyme. Worms were then washed four times with PBST for 10 min. Worms were incubated for 1 hr in blocking buffer on a roller. After blocking, the solution was replaced with fresh blocking buffer containing 1/50 affinity purified sheep Anti-Digoxigenin-POD Fab fragments and left overnight on a rocker at 4 ^{o}C. The protocol then follows the single FISH method using a rhodamine TSA solution (Appendix 4).

2.9.4 Automation

Towards the end of the project, an InsituPro VSi (Intavis), was purchased by the Molecular Laboratories, allowing automation of WMISH protocols. Several runs using the robot were trialled to optimise the protocols described above for colorimetric, single and double FISH methods. For an example of the output for an automated dFISH run, see Appendix 5. The most common adjustments included increased number and duration of wash steps due to a lack of agitation provided by the robot. Although successful reactions were

performed, consistent automated results were not achieved during the initial trials.

2.10 Immunohistochemistry

2.10.1 Synapsin staining

Synapsin is a broad neuronal marker that was used to stain the nervous system of *H. microstoma* to investigate potential localisation of gene expression within (or associated with) the nervous system. Fresh worm samples that had not come into contact with ethanol (as this alters the binding site of the anti-synapsin antibody) were permeabilised in 1% sodium dodecyl sulfate (SDS) for 1 hr (instead of proteinase K, as this destroys the snynapsin binding site) and then followed the single FISH method. Once the anti-DIG antibody had been removed and washed off, specimens were incubated with 100 mM sodium azide (to quench HRP activity before detecting anti-synapsin and prevent cross-reactivity of the tyramide solutions, as in dFISH). Once samples were washed with PBST to remove sodium azide, worms were incubated for 1-2 hr in blocking buffer. After blocking, the solution was replaced with fresh blocking buffer containing 1/200 anti-synapsin antibody (Developmental Studies Hybridoma Bank) and left overnight on a rocker at 4 ^{o}C. The antibody was removed and the worms washed four times in PBST. Specimens were incubated for 1 hr in blocking buffer and then replaced with goat-anti mouse HRP and left overnight on a

rocker at 4 ºC. The signal was then amplified following the TSA method with rhodamine TSA solution as described above.

2.10.2 DAPI counterstaining

4',6-diamidino-2-phenylindole (DAPI) is a fluorescent dye that stains AT rich regions of nuclear DNA. By fluorescently staining the nuclei of all cells, DAPI can be used as a morphological road-map of *H. microstoma* that allows the broader structure of the worms to be seen during FISH and immunohistochemical staining. All FISH, dFISH and synapsin prepared worms were counterstained with DAPI (Thermofisher) by incubating in a 4 ng/ml solution for 10 min on a roller and were then washed at least four times in PBST. Worms were transferred to 50% glycerol/PBST and allowed to settle and the solution replaced with 80% glycerol/PBSTx. Once the worms had settled and cleared, specimens were mounted on glass slides and stored at 4 ºC.

2.11 Imaging

2.11.1 Light microscopy

Images of non-fluorescently stained worms were taken using differential interference contrast (DIC) on a Leica DM5000 compound microscope with mounted DFC450 C digital camera and Leica Application Suite software. DIC microscopy uses polarised light to enhance the contrast in unstained

specimens. Using DIC after WMISH allows visualisation of the structures of the worm and see more clearly where gene expression is occurring.

2.11.2 Epifluorescence microscopy

FISH, dFISH and immunohistochemically stained specimens were first observed using epifluorescence. Light is passed through a filter cube, that contains an excitation filter, dichroic mirror and emission filter. The light first passes through the excitation filter that narrows the wavelength of the transmitted light to the wavelength of excitation for the fluorophore used. The mirror the reflects the filtered light through the objective to the specimen. This light is absorbed by the fluorophores of the stain which then emit back light of a longer wavelength (i.e. it fluoresces). The emitted fluorescent light passes back through the objective and the filter cube once more. The light first has to pass through the dichroic mirror that allows through emitted fluorescent light but acts as a barrier to any reflected excitation light. Next, the emitted light has to pass through an emission filter that will only let through light of the expected wavelength of the excited fluorophore. The filtered emitted fluorescent light is then directed to the eye piece or camera where it can be observed. FISH and dFISH specimens stained with fluorescein, rhodamine and DAPI were observed using a pE-300 LED light source (coolLED) mounted on a Leica DM5000 microscope equipped with L5, Rhodamine and DAPI/A filter cubes.

2.11.3 Confocal microscopy

Fluorescently stained worms were next observed and analysed using a Nikon Eclipse compound upright microscope with a Nikon A1-Si confocal microscope and Nikon Elements software. Lasers with wavelengths of 405 nm, 488 nm and 561 nm using the 'DAPI', 'FITC' and 'TRITC' laser lines were selected to acquire fluorescent confocal images. The 'transmitted detected' option was selected that allowed an image of the specimen to be taken using the transmitted light of the lasers. Once the specimen was in focus, a Z-stack was created by specifying the top and bottom planes of the worm by focusing through the specimen. The recommended number of steps (i.e. the number of images taken) was calculated by the Elements software, based on the thickness of the specimen and the magnification of the objective. Z-corrections throughout the stack were performed by adjusting the HV-gain and offset whilst moving through different focal planes. This was to reduce background and to keep signal strength constant and not under- or over-exposed due to (for example) the thickness of the specimen. To reduce bleaching of the specimens, pixel dwell was left at 2.3 with an image resolution of 1024 x 1024, pinhole size was selected at the recommended size (but was usually 1.0 or 1.2 AU) and no averaging was used. The lasers were run in a channel series (i.e. run sequentially) to reduce channel bleed through. Laser power was left generally low between 0.77 and 1.0. For larger scans, encompassing several fields of view, the above settings were adjusted to reduce bleaching and 'chequerboarding'. Pixel dwell was reduced to 1.0, 2x averaging was performed to reduce noise and the pinhole

was reduced to 0.8 and the overlap of fields was reduced to 5%. The images were stitched together using Elements. Resulting images were saved as Nikon ND2 files.

2.11.4 Post-processing

Image adjustments were made using the Fiji distribution of ImageJ software (Schindelin et al., 2012; Schneider et al., 2012). Maximum projections were created using the 'create z-stack' plugin. Cross-sections were created using 'orthogonal view' and by 'reslicing' the raw image and interpolating the image steps. A maximum projection of up to twenty new stacks of the resliced image resulted in the final cross-section reconstructions. Image colours were modified using lookup tables in Elements and brightness/contrast settings in Fiji. Images were converted to tiff and jpeg file formats and figures were prepared using GIMP (available at http://gimp.org) and Adobe Illustrator software (Adobe).

2.12 Development of an *in vivo* approach to RNAi for functional analyses

2.12.1 Synthesis of dsRNA and preparation of chemical inhibitors

RNAi probes were created for the posterior Hox gene *Post-2* (Pouchkina-Stantcheva et al. 2013) using the T7 MEGAscript Kit (Ambion). RNA was precipitated in alcohol with lithium chloride (as per kit instructions) and re-

suspended in 40 µL DEPC water. The concentration of the RNA was determined using a NanoDrop.

The Wnt pathway inhibitor IWP-3 was dissolved in dimethyl sulfoxide (DMSO) to form a stock solution of 1 mM/ml which was further diluted to working concentrations of 20 µM/ml + 1% DMSO and 40 µM/ml + 1% DMSO. DAPT (an inhibitor of the Notch pathway) was dissolved in DMSO to create a 25 mM stock solution and diluted to working concentrations of 50, 75 and 100 µM/ml + 1% DMSO.

2.12.2 Preparation of glass micropipettes

Using a P-2000 micropipette puller (Sutter Instrument Company), 0.5 mm glass tubes were pulled to form micropipettes using the following settings: heat – 350; pull – 150; velocity – 50 and delay – 50. The micropipettes are sealed at the end due to the heat from the laser. Therefore, prior to use, the end of the micropipette must be snapped using a pair of fine forceps, creating a sharp, tapered needle. See Chapter 6 for a detailed protocol of the injection methods developed to investigate RNAi in *H. microstoma*.

Chapter 3

The Notch pathway in *Hymenolepis microstoma*

3.1 Introduction

Segmented bodies are a characteristic feature of many animals. The Notch pathway (Fig. 3.1) has emerged as a key player in the establishment of metameric units and has been dubbed part of the 'segmentation' or 'oscillator' clock (Palmeirim et al., 1997; Jouve et al., 2002; Pourquié, 2003). This clock mechanism is coordinated by Notch signalling and results in waves of expression that regulate cyclical segment formation. Whilst Notch signalling is not involved in the segmentation of *Drosophila melanogaster* (Tautz, 2004), recent studies have implicated Notch in the segmentation of other invertebrates (Stollewerk et al., 2003; Schoppmeier and Damen, 2005; Chipman and Akam, 2008; Pueyo et al., 2008; Rivera and Weisblat, 2009; Mito et al., 2011; Chesebro et al., 2012; Williams et al., 2012; Eriksson et al., 2013; Liu, 2013). Although strobilation is likely to have evolved independently in tapeworms (Seaver, 2003), Notch signalling may have been co-opted in the process due to its being an evolutionarily conserved cell signalling system that coordinates boundaries between repeated regions.

3.1.1 Notch discovery

The name, Notch, comes from the notches formed along the wing margins of mutant flies that were first observed by Dexter (1914). A few years later, the condition was found to be heritable and was described as an X-linked mutation that caused notches to form on the wings of females and embryonic death in males (Morgan and Calvin, 1916). In the mid 1930's, Poulson

described the 'neurogenic' phenotype of *D. melanogaster* embryos lacking *Notch* and was one of the first to associate links between genes and embryogenesis (Poulson, 1937). By the 1980's the molecular analysis had begun and *Notch* was sequenced in both *D. melanogaster* (Wharton et al., 1985; Kidd et al., 1986) and *Caenorhabditis elegans* (Greenwald, 1985). Investigations have since shown *Notch* to be a component of a signalling pathway that is highly conserved across the Metazoa (Gazave et al., 2009).

A study by Gazave et al. (2009), that investigated the presence of components of the Notch pathway in eight eukaryote clades, found that most animals possess a single *Notch* ortholog. This is not a universal trait, however, as vertebrates were found to possess between two and four copies, whilst the nematode *C. elegans* has two (*LIN-12* and *GLP-1*) (Gazave et al., 2009). The *Notch* genes themselves encode proteins that act as receptors. Notch proteins all have highly similar architecture, containing several (but not necessarily all of the following) domains: a signal peptide; Epidermal Growth Factor (EGF) repeats; the Lin-Notch repeat (LNR) or Notch domain; the NOD and NODP domains; a transmembrane region; a RAM23 domain; a series of Ankyrin repeats and finally a proline, glutamine, serine, threonine-rich (PEST) domain (Gazave et al., 2009). The ligands of the pathway belong to the Delta/Serrate/LAG-1 (DSL) family comprising of the genes *Delta* (*Dl*) and *Serrate* (*Ser*) (*Dl* and *Jagged* (*Jag*) in vertebrates and *LAG-2* and *APX-1* in *C. elegans*). DSL proteins also have several domains: a signal peptide; and MNLL domain; a DSL domain; EGF repeats; in the case of Ser/Jag a vWC domain and finally, a transmembrane region.

As Notch pathway ligands and receptors are membrane bound, signalling occurs between neighbouring cells in direct contact (Artavanis-Tsakonas et al., 1999). The only exception is found in nematodes, which have one secreted DSL protein alongside three membrane-bound ligands (Chen and Greenwald, 2004).

3.1.2 Mechanism of action

For a general overview of the mechanism of action for the Notch pathway, see Fig. 3.1. The Notch protein must be modified before moving to the surface of the signalling cell (Fortini, 2009). These modifications help to facilitate binding of Notch to its ligands and are also involved in the modulation of the pathway. Whilst still in the endoplasmic reticulum, carbohydrates are added to the EGF repeats of Notch. Carbohydrates are added first by O-Fucoslytransferase (Ofut/Pofut) (Ge and Stanley, 2008; Stahl et al., 2008) and then by Rumi/Poglut (Acar et al., 2008). It has been suggested that *Rumi* acts as a positive regulator by promoting extracellular cleavage of Notch (Acar et al., 2008).

During translocation to the cell membrane, Notch is cleaved three times. The first (S1) cleavage occurs within the Golgi apparatus and is executed by the protease Furin. Cleavage results in a heterodimer, consisting of the extracellular domain (NECD) and intracellular domain (NICD). In flies, S1 cleavage is not necessary for successful Notch signalling (Bush et al., 2001; Kidd and Lieber, 2002). Whilst still in the Golgi apparatus the two domains

[Figure removed due to copyright]

Figure 3.1 The Notch signalling pathway. See image text and chapter for a more detailed description of the pathway's mechanism of action and factor interactions. (From Ilagan and Kopan, 2007)

undergo further carbohydrate modification by Fringe and GXYLT/Shams. Fringe promotes Notch/Dl binding over Notch/Ser, as the addition of GlcNAc inhibits the interaction between Notch and Ser ligands (Brückner et al., 2000; Okajima et al., 2003). However, *Fringe* is not found in all taxa examined so far (Gazave et al., 2009) and so may not be crucial to Notch signalling. GXYLT/Shams, elongates the glucose residues added by Rumi (Lee et al., 2013). After these after modifications within the Golgi apparatus, the Notch receptor translocates to the cell membrane.

If Notch signalling is not required, the receptor is ubiquinated by several E3 ubiquitin ligases including *Nedd4*, *Suppressor of deltex* (*Su(dx))/Itch*, *Archipelago* (*Ago*)*/Fbxw7* and *Cbl* (Le Bras et al., 2011). Other inhibitors of the pathway include *Numb* and *Deltex*. By binding to Notch, Numb helps to promote its degradation, by acting as a scaffold that E3 ubiquitin ligases can bind to, enabling them to break down Notch (Frise et al., 1996; McGill and McGlade, 2003; McGill et al., 2009).

If Notch signalling is required, the receptor binds to a DSL ligand expressed on the signalling cell. Binding brings about a conformational change of the NEDC, which exposes residues that can be cleaved by the metalloproteases Kuzbanian/ADAM10 (Kuz) and ADAM17/TACE (TACE), also known as S2 cleavage (Brou et al., 2000; Lieber et al., 2002). The release of the NECD results in endocytosis of the DSL ligand by the signalling cell. The endocytosis and recycling of ligands is triggered by two more E3 ubiqitin

ligases: Mindbomb (Mib) and Neuralized (Neur), which can also actively degrade the DSL ligands (Le Bras et al., 2011).

The third and final cleavage (S3) of the NICD is performed within the cell membrane by the membrane-bound γ-secretase complex (De Strooper et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999). The γ-secretase complex is comprised of four proteins – Presenilin (Pres), Nicastrin, Anterior pharynx defective 1 (APH1) and Presenilin enhancer 2 (PEN2). After S3, the NICD is released into the cytoplasm where it translocates into the nucleus. There, NICD interacts with the *CSL*/*Ncor/SMRT/Histone Deacetylase* transcriptional complex. In the absence of the NICD, Suppressor of hairless (Su(H)) is bound to co-repressors (including Hairless, Groucho (Gro) and SMRT) within the transcriptional complex. Once the NICD is associated with *Su(h)*, the co-repressors disassociate, and the co-activators *Mastermind*, *Skip* and *Histone acetylase* (*Hac*) are recruited. Transcription of downstream targets, including genes belonging to the *Enhancer of split* (*E(spl)*) complex, is then activated.

The *E(spl)* complex includes the most well-known, direct transcriptional targets of Notch signalling (Greenwald, 1998; Artavanis-Tsakonas et al.,1999). Genes include those belonging to the *Hes*/*Hey* family of basic helix-loop-helix (bHLH) transcription factors and members of the *Bearded* (*Brd*) family (Lai et al., 2000). In *C. elegans*, a group of related bHLH transcription factors, the *Ref* family, are targets of Notch signalling rather that *Hes/Hey* genes (Neves and Priess, 2005).

3.1.3 Modulation and regulation of the Notch pathway

3.1.3.1 *Cis* and *trans* regulation

Notch signalling occurs through direct cell-cell contact between signal-sending and signal-receiving cells (Artavansi-Tsakonas et al., 1999). Both receptors (Notch proteins) and ligands (DSL proteins) are single-pass transmembrane proteins that are found on the cell surface. Interactions between ligands and receptors of neighbouring cells result in activation of Notch signalling and is referred to as *trans* activation. However, ligands can also interact with receptors that are expressed in the same cell as each other – *cis* inhibition (de Celis and Bray, 1997; Klein et al., 1997; Micchelli et al., 1997; Miller et al., 2009; Sprinzak et al., 2010; del Álamo et al., 2011. This can be through the inhibition of the receptor by the ligand, mutual inhibition or even the inhibition of the ligand by the receptor (del Álamo et al., 2011). All *cis* interactions though act to inhibit Notch, reducing its capacity to receive signals from neighbouring cells.

3.1.3.2 Inhibitory and inductive Notch signalling

Notch signalling can generally be divided into two routes of action: "inhibitory" and "inductive" signalling (reviewed in Lai, 2004; Bray, 2006). Lateral inhibition is, probably, the most prominent modality and occurs between groups of cells with similar developmental potentials. In this situation *Notch* acts as a "switch", resulting in binary cell fate decisions.

Reciprocal, mutual inhibition between equipotent cells will resolve over time with one cell committing to a certain fate (via a feedback loop) which then inhibits surrounding cells from also adopting this fate. Selection of a single fate by one cell can be amplified beyond its neighbouring cells, producing "salt and pepper" patterning within a field of cells though feedback loops. One example of lateral inhibitory signalling can be seen in *Hydra*, where Notch signalling helps maintain the border between tentacles and head (Münder et al., 2013).

Inductive signalling tends to occur between cell populations with different developmental potentials and results in the formation of a developmental boundary. During inductive signalling, the signalling cell activates *Notch* in the receiving cell, inducing a new cell fate type in response that acts as a boundary between the initial two cell types. A well-known example of this can be seen during development of the dorsoventral boundary of the fly wing imaginal disc (Fleming et al., 1997; de Celis et al., 1996; Bray, 2006).

3.1.3.3 Post-translational modification

Beyond regulation of the Notch pathway through *cis* and *trans* interactions, signalling can also be regulated through post-translational modifications. Several pathway components are integral in post-translational modification. Factors that are involved in ubiquitination, phosphorylation and glycosylation during activation of the pathway (see above) are critical in the regulation of Notch signalling (Haines and Irvine, 2003; D'Souza et al., 2008; Fortini,

2009; Le Bras et al., 2011). For example, some of these modifications can help to promote degradation of Notch (Frise et al., 1996; McGill and McClade, 2003; McGill et al., 2009) or DSL ligands (Le Bras et al., 2011).

3.1.3.4 The pleiotropic nature of Notch signalling

Notch signalling is extraordinarily pleiotropic, involved in the cell fate determination of most tissues (Guruharsha et al., 2012; Hori et al., 2013). The developmental outcomes of Notch are highly context-specific, and the way in which it interacts with other pathways can result in different fates. For example, the same level of signal in one tissue type can result in cell death, whilst in another promote cell proliferation (Hori et al., 2013). One particular example of the pleiotropic nature can be seen in its role in the development of various cancers. Notch signalling can be oncogenic, promoting tumour growth in the haematopoietic system and breast tissue (South et al., 2012; Kushwah et al., 2014). However, in skin cancers (and possibly vascular cancers) Notch acts as a tumour suppressor (South et al., 2012; Kushwah et al., 2014).

3.1.4 Notch and segmentation

Notch signalling plays an important role during segmentation and somitogenesis and is involved in the synchronisation of the segmentation clock (Palmeirim et al., 1997; Jiang et al., 2000; Pourquié, 2003; Aulehla and Herrmann, 2004; Liao and Oates, 2016). Although an oscillatory wave of

Notch signalling has not clearly been shown functionally in arthropods, Notch is involved in the segmentation of a number of arthropods including crustaceans, spiders, myriapods and insects (Stollewerk et al., 2003; Schoppmeier and Damen, 2005; Chipman and Akam, 2008; Pueyo et al., 2008; Chesebro et al., 2012; Williams et al., 2012; Eriksson et al., 2013; Liu, 2013). In these arthropods, transcripts of pathway components (including *Notch*, *Dl*, *Ser*, *Su(h)*, *Fringe* and *Gro*) are expressed in sequential stripes along segment boundaries. Knockdown of *Notch* via RNAi and treatment with DAPT in the cockroach, *Periplaneta americana*, results in segment phenotype defects affecting size, width and shape and in extreme cases, segmentation can be halted altogether (Pueyo et al., 2008). Notch signalling has also been shown to interact with the Wnt pathway in the spider, *Parasteatoda tepidariorum* (McGregor et al., 2008). More recently, this interaction between Delta-Notch signalling and Wnt was found to be pleiotropic, required for the context-specific expression or repression of *Wnt8* in the posterior or anterior segment addition zone respectively (Schönauer et al., 2016). Where *N* and *Dl* repress *Wnt8*, it is thought that downstream targets (*Even-skipped*, *Runt-1* and *Caudal*) are activated, resulting in segment formation (Schönauer et al., 2016).

However, Notch signalling is not universally required for segment formation in arthropods, including *D. melanogaster* (Tautz, 2004; Peel et al., 2005). *Dl* is expressed in stripes along segments in the cricket, *Gryllus bimaculatus*, but is not necessary for segmentation (Kainz et al., 2011; Mito et al., 2011). Inhibition of *Dl* and *Notch* leads to severe patterning defects after the

morphological appearance of segments, indicating instead that Notch signalling is involved in post-segmentation patterning (Kainz et al., 2011; Mito et al., 2011). In *Tribolium* spp., inhibition of *Hairy* implies that Notch signalling is involved in the patterning of segments rather than their specification (Aranda et al., 2008). Whilst the role of Notch signalling in the patterning of segments after their morphological appearance (rather than in the direct establishment of segments) is echoed in the honey bee, *Apis mellifera*. In *A*. *mellifera*, *Delta* is expressed in a striped pattern, indicating an involvement in establishing segments. However, after inhibition of Notch signalling through RNAi or chemical inhibition, deformed segments are still formed (although with pattering defects) (Wilson et al., 2010).

Literature relating to a role for Notch signalling in lophotrochozoans is limited, but the pathway does appear to be involved during segmentation of the leech, *Helobdella robusta* (Rivera and Weisblat, 2009). *Hes* is a confirmed target of Notch signalling in annelids (Rivera and Weisblat, 2009) and can therefore be used as a readout for Notch signalling. Expression of *Hes* in *H*. *robusta* peaks as segmental founder cells are produced and both *Hes* and *Notch* are expressed in an oscillatory manner (Song et al., 2004; Rivera et al., 2005), thus resembling the clock/wavefront of vertebrate somitogenesis. Coupled with this, loss of both *Notch* and *Hes* leads to segmentation defects indicating a role of Notch signalling in the patterning of segments (Rivera and Weisblat, 2009). However, in another lophotrochozoan, *Capitella* sp. I, not obvious role could be found for Notch signalling in the segmentation process (Thamm and Seaver, 2008). Despite not being segmented, understanding

the role of Notch signalling in planarians would help to uncover the role it plays in tapeworms. Unfortunately, no research on Notch signalling is available in planarians.

3.2 Results

3.2.1 Most of the core components of the Notch pathway are conserved in *Hymenolepis microstoma*

3.2.1.1 Notch receptor genes

The first categories of Notch pathway genes investigated were the Notch receptors themselves. Initial BLAST searches of the genome, alongside mining of the online web tool WormBase ParaSite (Howe et al., 2015) identified as many as four *Notch* orthologs in *H. microstoma* (data not shown). However, detailed investigations using reciprocal BLAST searches and analysis of predicted protein domain architecture using CDD, InterPro and SMART (Schultz et al., 1998; Mitchell et al., 2014; Letunic et al., 2015) narrowed this down to two orthologs (Fig. 3.2 and Table 3.1). This number is comparable to that seen within the rest of the Metazoa - *C. elegans* has two whilst there are as many as four in some vertebrates and only one in dipterans (Gazave et al., 2009), indicating no gene expansion or loss within the flatworms. Indeed, BLAST searches also identified two *Notch* orthologs present in all the flatworm genomes available. Gene trees show that this

gene duplication was an ancestral occurrence within the Platyhelminthes (data not shown).

3.2.1.2 Notch receptor-associated genes

Several genes are involved in the regulation of Notch through cleavage (*Furin*, *Kuz*, *Tace*, *Pres*, *APH*, *Nicastrin*, and *Pen2*); ubiquitination (*Deltex*, *Ago/Fbxw7*, *Itch/Nedd4* and *Cbl*); modification via carbohydrate transferases (*Rumi*, *Fringe*, *Ofut/Pofut*, and *Shams*) and finally inhibition (*Numb*, *Spdo* and *Lnx*). *H. microstoma* possesses orthologs of all the genes involved in the cleavage of Notch, including two *Furins* (Fig. 3.3 and Table 3.1). Orthologs of both metalloproteases involved in S2 cleavage - *Kuz* and *Tace* are present (Fig. 3.3), though several more ADAM-like genes are also found in the genome (data not shown). All four members of the γ-secretase complex (*Pres*, *APH*, *Nicastrin*, and *Pen2*) are present. The number of cleavage genes in *H. microstoma* indicates no loss or expansion within the tapeworms.

Unlike the cleavage factors, not all genes involved in the regulation of Notch by other means are present in the *H. microstoma* genome. No ortholog of *Deltex* could be found, as is also the case in nematodes and the few Lophotrochozoans so far investigated (Gazave et al., 2009). *Deltex* is thought to be an activator of Notch signalling and is found in the sponge *Amphimedon queenslandica* and outside the Metazoa - in choanoflagellates, but are missing or only partially present in placozoa (Gazave et al., 2009). *Deltex* is also absent in *Hydra* and *C. elegans* and some diagnostic domains

are missing from *Deltex* genes in the leech (*H. robusta*), giant clam (*Lottia gigantea*) and the starlet sea anemone (*Nematostella vectensis*). Combined, these losses suggest that there may be significant sequence divergence or even the loss of *Deltex* in some phyla. As other E3 ubiquitin ligases such as *Nedd4* (of which there are three), *Clb* (three) and *Ago* (one) are present, it may be that one of these has filled the role of *Deltex* within these phyla. The number of *Nedd4* and *Cbl* orthologs is comparable to that of vertebrates, as is *Ago*.

Only one gene involved in the post-translational modification of Notch could be identified: *Ofut/Pofut* (for which there are no diagnostic domains). No ortholog could be identified for *Rumi*, *Fringe* or *Shams*. This is similar to observations in nematodes, which only possess *Pofut*. The loss of *Fringe* is of particular interest as it biases binding of Notch with Dl over Ser. However, it is also absent from *H. robusta* and *C. elegans* (Gazave et al., 2009). Instead, other mechanisms must be in place to regulate the binding of Notch to one ligand type over another. The loss of the three enzymes *Rumi*, *Fringe* and *Shams* suggests that either post-translational modification is not necessary to activate Notch activity in tapeworms or, more likely, that other modification mechanisms are in place, such as other carbohydrate transferases.

Finally, of the genes involved in the inhibition of *Notch*, an ortholog of *Numb* and *Spdo* were found in *H. microstoma*. The absence of *Lnx* supports the idea that this protein is only found in vertebrates (Lai, 2002).

3.2.1.3 Notch Ligands

H. microstoma has seven DSL ligands, all identifiable through the presence of a DSL domain. As was the case with initial *Notch* searches, more than seven potential ligands were flagged, but further investigations showed that these did not possess the DSL domains indicative of Notch ligands and instead only contained repeated EGF domains. Analysis of the domain architecture of the predicted proteins points to one *Hmic-Ser/Jag* gene, due to the presence of a von Willebrand Factor, Type C domain (Fig. 3.2). The remaining six of the seven DSL ligands are *Dl* genes, which are shorter (as they typically are) than the single *Hmic-Ser/Jag* gene and also lack the von Willebrand Factor domain. All *H. microstoma* DSL ligands have a transmembrane domain, as found in vertebrates and are not secreted, unlike nematode ligands (Chen and Greenwald, 2004). Like *C. elegans* however, *H. microstoma* ligands do not have a recognisable Notch-terminal MNLL domain (Gazave et al., 2009).

3.2.1.4 DSL ligand-associated genes

The E3 ubiquitin ligases *Mib* and *Neur* are responsible for controlling DLS ligands, through their involvement in ligand endocytosis, maturation and degradation. There are two *Mib* orthologs in *H. microstoma*, as there are in vertebrates and flies (Gazave et al., 2009), but no ortholog of *Neur* could be identified. This is interesting as *Neur* is thought to have evolved within the

Bilateria (Gazave et al., 2009), indicating possible loss of the gene within tapeworms.

3.2.1.5 Transcription and co-transcription factors

The core transcription factor of the Notch pathway is *Su(h)*. Like most metazoans, *H. microstoma* possess one *Su(h)* ortholog (Fig. 3.3), as do all other flatworms studied in this survey. The literature also indicates four other genes belonging to the transcriptional complex: the co-activators *Skip* and *Mam* and the co-repressors *Gro* and *Hairless*. Interestingly, *H. microstoma* possess one *Skip* ortholog but no *Mam*. *Mam* is present throughout the Metazoa, including the Cnidaria, however, a survey by Gazave et al., (2009) highlighted that *Mam* is also missing in the annelid, *H. robusta*, and the mollusc, *L. gigantea*. Its absence from these three phyla suggests *Mam* was lost from the last common ancestor of lophotrochozoans. *H. microstoma* has two orthologs of the co-repressor *Gro*, similar to the situation observed in most other metazoans, exceptions including vertebrates that have four and dipterans and nematodes which possess one. No ortholog could be found for most other metazoans, exceptions including vertebrates that have four and dipterans and nematodes which possess one. No ortholog could be found for the other co-repressor, *Hairless*. This gene has only been reported in insects so far though and its absence in tapeworms supports the hypothesis that it is a fast-evolving gene unique to the insects (Maier, 2006). Finally, an ortholog of *Smrt*, which is also part of the transcriptional complex, acting as a corepressor, is present in *H. microstoma.*

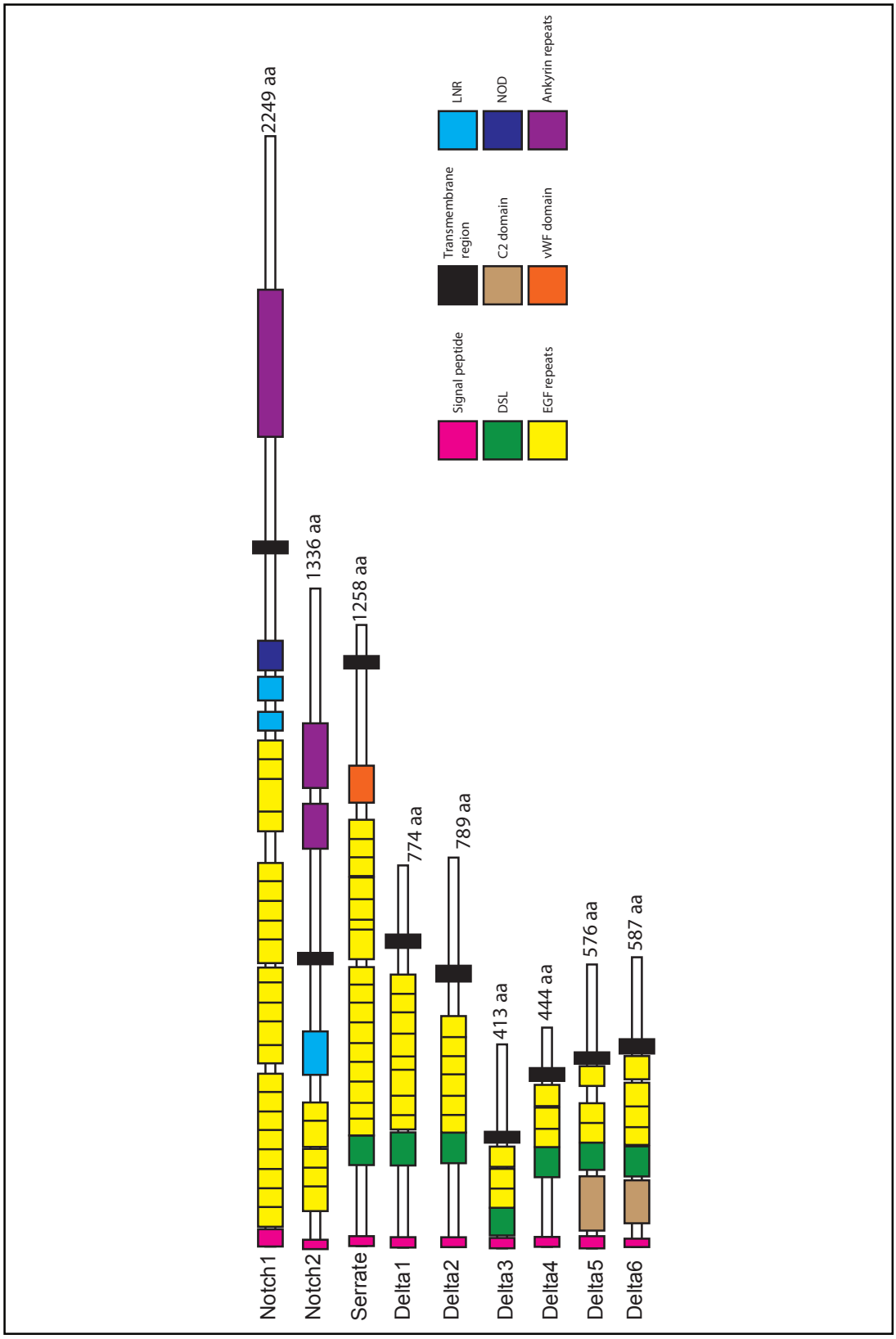

Fig 3.2 Structure of *Hymenolepis microstoma* Notch pathway ligands and receptors. The domain organisation of proteins belonging to the Notch pathway. Smart and InterPro were used for domain analysis and the major domains used to define the proteins are shown. The number of amino acids in each protein is also given.

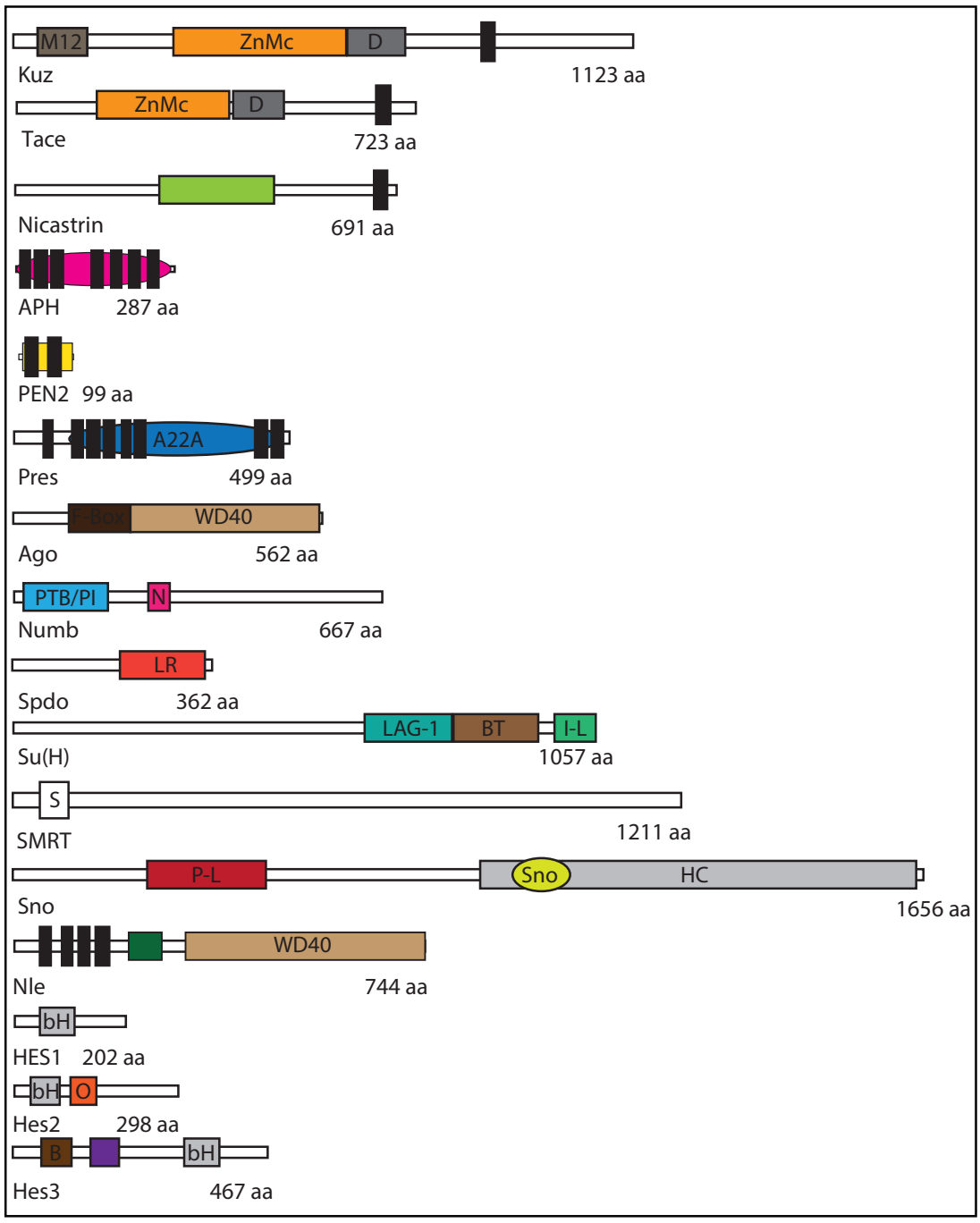

Fig 3.3 Structure of *Hymenolepis microstoma* Notch pathway factors. The domain organisation of proteins belonging to the Notch pathway. Smart and InterPro were used for domain analysis and the major domains used to define the proteins are shown. The number of amino acids in each protein is also given. Diagnostic domains: M12 = peptidase M12B propeptide, ZnMc = zinc-dependent metalloprotease, D = disintegrin, A22A = peptidase A22A, light green = nicastrin, pink = gamma secretase subunit APH-1, yellow = presenilin enhancer-2 subunit, LR = leucine-rich repeat, dark green = NLE, WD40 = WD40 repeat containing domain, PTB/PI = phosphotyrosine-binding/ phosphotyrosine interaction domain, N = Numb domain, S = SANT, LAG1 = LAG-1, BT = beta-trefoil, I-L = immunoglobulin-like fold, bH = basic

helix-loop-helix, B = bromodomain, purple = NET, O = Orange domain, P-L = P=loop NTPase, Sno = Strawberry notch domain, HC = Helicase C, F-Box = F-box domain, black = transmembrane region.

3.2.1.6 Transcriptional targets

The *Hes/Hey* family of genes are the main downstream transcriptional targets of Notch signalling, alongside members of the *Brd* and *Ref* families (Lai et al., 2000), and are used as 'readouts' for Notch signalling. There are three *Hes* genes in *H. microstoma*. This contrasts with the large expansion of the family in vertebrates and flies that have 13 members, but is more in line with the single ortholog (*Lin-22*) in nematodes. This indicates a small expansion within tapeworms but not nearly so great as in vertebrates and flies. No ortholog could be identified for members of either the *Brd* family which is thought to be insect specific or the *Ref* family that appears to be unique to the Nematoda (Lai et al., 2000; Neves and Priess, 2005).

3.2.1.7 Miscellaneous factors

Finally, there are a few factors within the Notch pathway whose roles are still not entirely understood, but seem to be involved in pathway modulation. *Notchless* (*Nle*) is thought to act as a regulator, inhibiting *Notch* activity (Royet et al., 1998). *Strawberry notch* (*Sno*) is another gene thought to be involved in modulating Notch activity, possibly through interactions with the transcription factor *Su(h)*. There are clear orthologs for both *Nle* and *Sno* in tapeworms (Fig. 3.3).

3.2.2 RNA-Seq data

RNA-Seq data was made available for approximately the mid stages of larval development, whole adults, the scolex and neck, mature sections of the strobila and finally gravid strobila (Tsai et al., 2013). RNA-Seq data (for these five developmental stages) for genes identified as belong to the Notch pathway can be seen in Table 3.1.

Looking at the RNA-Seq data (Table 3.1), only 7 genes have any statistical change in FPKM expression levels: *Hmic-Dl1*; *Hmic-Dl4*; *Hmic-Dl5*; *Hmic-Ser*; *Hmic-Hes3*; *Hmic-Tace* and *Hmic-Smrt*. Of these, four are upregulated in adults, one in larvae and one in the scolex/neck. These are of particular interest due to the distinct developmental features during these life stages.

The data show that *Hmic-Ser* is upregulated in larvae when compared to adults, and that there are higher levels of expression in the scolex/neck compared to the rest of the adult worm (Table 3.1). This upregulation in the scolex and neck and larvae potentially indicates a role during both strobilation and larval development. Whilst the expression levels of *Hmic-Ser* imply an increased role in larvae, there generally seems to be higher expression of *Dl*s in adults. *Hmic-Dl1*, *Hmic-Dl4* and *Hmic-Dl5* are all upregulated in adult *H. microstoma* adults, compared with larvae. There is also increased expression in the end regions of the worm of both *Hmic-Dl1* and *Hmic-Dl5* implying a role in embryogenesis or egg maturation. *Hmic-Dl3* and *Hmic-Dl6* expression is more consistent across the adult worm. They are

Table 3.1 Notch pathway factors in *Hymenolepis microstoma*. *H. microstoma* Notch pathway factors, their predicted gene model numbers and the length of predicted proteins in base pairs. RNA-Seq data for each gene model is given in the number of reads per kilobase per million mapped reads (RPKM) for four stages of development – mid larval stages, whole adult, the scolex/neck, mid (i.e. mature segments) and end (i.e. gravid segments). Differential expression between regions is given (from Tsai et al., 2013).

Gene	Gene model	Length (bp)	FPKM values					Differential expression			
			Larva	Whole adult	Scolex/ neck	Mid regions	End regions	Larva vs whole adult	Scolex/neck vs mid	Scolex/neck vs end	Mid vs end
Hmic-Notch1	HmN_000653600	6747	21.5	39.9	9	15.6	10.3	ND	ND	ND	ND
Hmic-Notch2	HmN_000853800	4008	34.7	33.7	13.4	10.2	8.5	ND	ND	ND	ND
Hmic-Dl1	HmN_000155700	942	5.8	68.5	12.9	19.9	64.4	UP	ND	UP	ND
Hmic-Dl2	HmN_000605500	2367	29.4	14.2	17.2	6.8	11	ND	ND	ND	ND
Hmic-Dl3	HmN_000714400	1239	51.1	106.4	50.1	69.7	68.3	ND	ND	ND	ND
Hmic-Dl4	HmN_000734400	1332	14.7	81.5	34.1	41.1	42.1	UP	ND	ND	ND
Hmic-Dl5	HmN_000376400	1761	15.3	97.5	51.8	63.3	81.6	UP	ND	UP	ND
Hmic-Dl6	HmN_000714300	1725	42.7	62.7	39.5	38.9	33	ND	ND	ND	ND
Hmic-Ser	HmN_000639400	3774	83.6	8.9	14	2.7	1.8	DOWN	DOWN	DOWN	ND
Hmic-Su(H)	HmN_000214100	3174	30.1	68.4	27.1	30.5	31	ND	ND	ND	ND
Hmic-HES1	HmN_000974100	606	0	0.4	0.1	0.4	0.2	ND	ND	ND	ND
Hmic-HES2	HmN_000343800	894	6.4	7.6	3	8.8	4.5	ND	ND	ND	ND
Hmic-HES3	HmN_000973500	1401	0	25.4	0.4	53	3.9	ND	UP	UP	DOWN
Hmic-Pres	HmN_000031500	1497	49.4	67.4	45.6	56.5	33.5	ND	ND	ND	ND
Hmic-Spdo	HmN_000234700	1086	158.1	346.1	315.3	250.5	293.4	ND	ND	ND	ND
Hmic-Ofut	HmN_000485500	1293	40	32.6	20.2	23.9	22.7	ND	ND	ND	ND
Hmic-Numb	HmN_000511800	2001	41.8	78.1	59.9	49	53.8	ND	ND	ND	ND
Hmic-Kuz	HmN_000907900	3369	20.4	40.5	15.7	25.4	23	ND	ND	ND	ND
Hmic-TACE	HmN_000264000	594	0.7	61.4	1.1	146.7	11.5	UP	UP	UP	DOWN
Hmic-Nicastrin	HmN_000242800	2073	67.7	41.6	43.7	30	31.4	ND	ND	ND	ND
Hmic-Pen2	HmN_000694400	297	37.9	44.6	9.9	8.6	7.3	ND	ND	ND	ND
Hmic-APH1	HmN_000691700	861	27.7	109	62.7	74.9	61.9	ND	ND	ND	ND
Hmic-SMRT	HmN_000838900	3633	28.8	48.3	11.6	35.1	15.3	ND	UP	ND	DOWN
Hmic-Nle	HmN_000357200	2232	27.7	38	28.6	29.2	22.8	ND	ND	ND	ND

therefore unlikely to be involved in H. *microstoma* strobilation. The results indicate a possible division of labour within the DSL ligands with *Hmic-Ser* playing a more prominent role during larval development and strobilation whilst *Hmic-Dl1*, *Hmic-Dl4* and *Hmic-Dl5* are more involved in sexual development, maturation and reproduction. Both *Hmic-Dl4* and *Hmic-Dl5* are upregulated in adults compared with larvae, although the expression levels of both these genes are fairly consistent across the entire worm, so it would seem unlikely that they would have a role during strobilation.

The expression levels of the downstream transcriptional targets of Notch signalling *Hmic-Hes1* and *Hmic-Hes2* are both extremely low and consistent in adult worms. *Hmic-Hes3*, however, is upregulated in adults compared with larvae and is more strongly expressed in the mid-regions of adults. The upregulation of a target of Notch signalling implies *Notch* is particularly active during maturation and development of gonads, possibly during processes such as oogenesis, even though *Notch* levels themselves are not high. There is an apparent division of labour between adult and larval HES activity: *Hmic-Hes1* and *Hmic-Hes3* are not expressed in larvae whilst *Hmic-Hes2* is active in larvae (although still at extremely low levels).

The expression of most genes within the pathway shows few statistical differences. Notch signalling is important during various life history and is involved in the coordination of many developmental processes (Artavanis-Tsakonas, 1999; Bray, 2006; Hori et al., 2013). The adult tapeworm undergoes many of these processes simultaneously (although regionalised).

As such, it would perhaps be more unexpected if there were more differences in gene expression across the worm. It is likely that small, localised modifications of the pathway lead to bigger changes in the output of Notch signalling. Visualisation of the tissues expressing Notch factors will be more informative in inferring their role during tapeworm development.

3.2.3 Expression analysis

3.2.3.1 Receptors

In adults, expression of *Hmic-Notch1* is observed in the neck in a strong central punctate stripe, surrounded by median and lateral punctate stripes (Fig. 3.4 A-D). The central foci are likely to represent expression of *Hmic-Notch1* during later development of the genital primordia (Fig. 3.4 D). During early strobilation, *Hmic-Notch1* expression is still observed during maturation of the genital primordia. In cross-section (Fig. 3.4 D), the foci of the median stripes mirror the location of the median nerve cords (Fig. 1.5). Once the neck has ended and strobilation begins, this expression ceases (Fig. 3.4 A-B). Towards the end of the neck, as strobilation starts, faint ladder-like punctate stripes can be seen when viewed using FISH (Fig. 3.4 B). Under higher magnification, it is apparent that this expression is found towards the posterior of the newly forming segments (Fig. 3.4 E). In cross-section, this expression appears as a ring in the cortex (Fig. 3.4 F). The ladder-like expression pattern is maintained along the length of the rest of the strobila, along the leading edge (posterior) of every segment (Fig. 3.4 I). As segments

develop, expression of *Hmic-Notch1* is observed in the seminal receptacle (Fig. 3.4 G-I). Once segments have fully matured, *Hmic-Notch1* is expressed centrally, in the ovary of *H. microstoma* (Fig. 3.4 J). As segments become gravid, expression of *Hmic-Notch1* is within the uterus (Fig. 3.4 K). Whole-mount staining of the uterus appears granular and when observed under higher magnification, it becomes apparent that *Hmic-Notch1* is expressed in discrete foci within the developing embryos (Fig. 3.4 L).

Hmic-Notch2 is more universally expressed, although weakly (Fig. 3.5 A). The general diffuse expression of *Hmic-Notch2* echoes RNAseq data (Table 3), which does not show any particularly strong or differential expression. When expression is observed using FISH, it seems that this universal staining is actually expression within cells in the cortex, close to the tegument (data not shown). In both immature and mature segments, *Hmic-Notch2* expression is stronger around the genital pore (Fig. 3.5 B-E). In mature and gravid segments, *Hmic-Notch2* is found in the uterus and embryos.

3.2.3.2 Ligands

The expression pattern of *Hmic-Ser* is in line with the RNA-Seq data (Table 3), with increased expression in the scolex and neck (Fig. 3.6). In the neck, *Hmic-Ser* is limited to granular, lateral stripes (Fig. 3.6 A-B). This pattern follows the position of the longitudinal nerve cords in the neck (Fig 1.4). Expression is strongest in the neck, becoming weaker as segments mature.

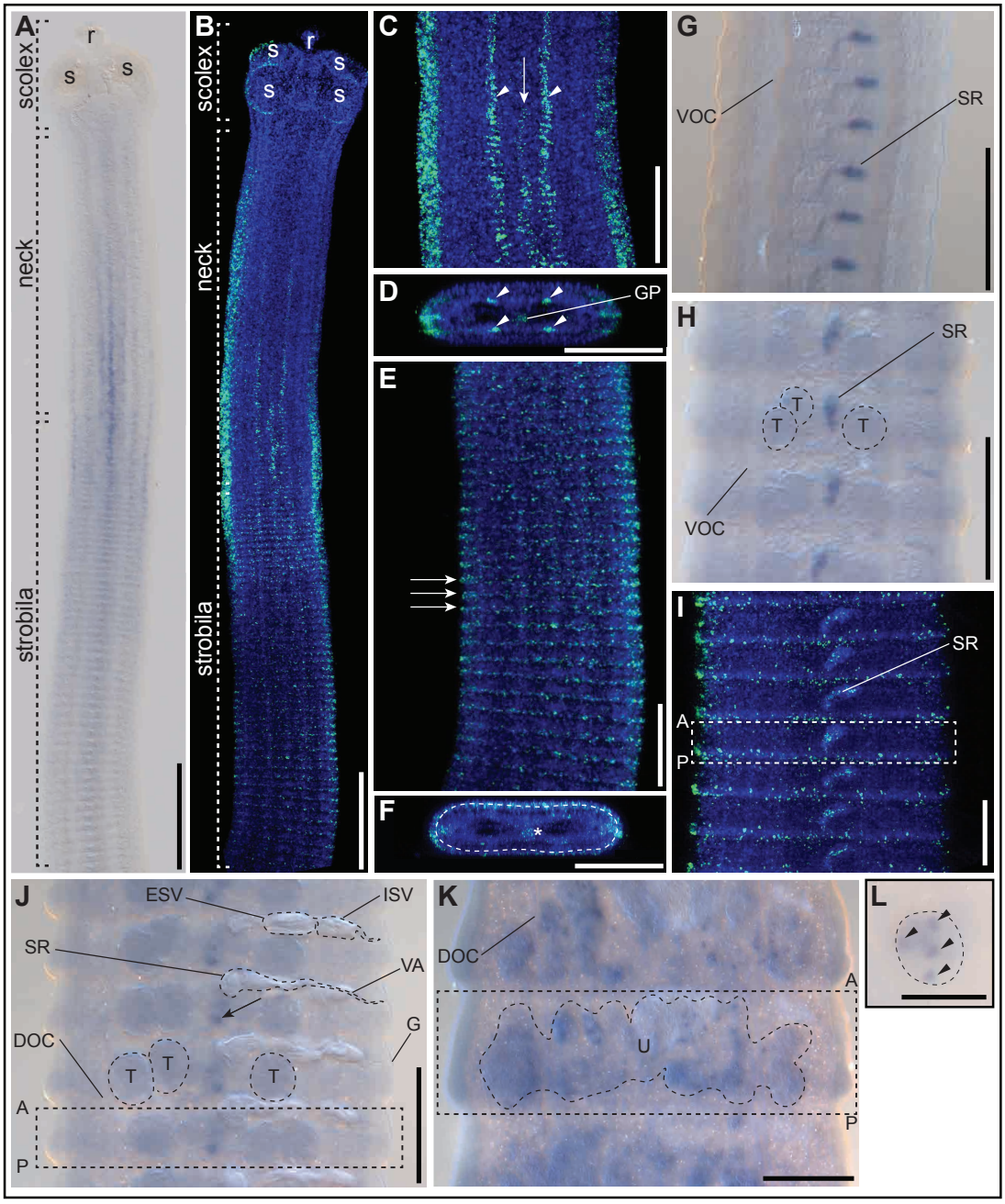

Figure 3.4 Expression of *Hmic-Notch1* in *Hymenolepis microstoma* adults. A) Colorimetric ISH and B) FISH of the adult worm. C) Maximum projection image of expression in the late neck and D) the late neck in cross-section. Arrow heads point to median stripes of expression, close to the median nerve cords whilst the arrow indicates a central stripe of expression during later development of the genital primordia within the neck. E) Expression within the early strobila. The arrows point to a punctate ring of expression within every segment. F) Cross-section of *Hmic-Notch1* expression in the early strobila. A punctate ring of expression is observed within cortical tissue. The asterisk indicates a central node of expression within the developing genital primordia. Colorimetric ISH within G) immature segments and H) maturing segments. I) FISH of maturing segments showing

the continued punctate ring of expression towards the posterior of each segment. Colorimetric ISH of J) mature segments (the arrow points to central expression within the ovary), K) gravid segments and L) a developing embryo within the uterus of a gravid segment. The arrowheads highlight discrete foci of expression within the embryo. Dashed boxes designate one segment. A = anterior, DOC = dorsal osmoregulatory canal, ESV = external seminal vesicle, G = genital pore, GP = genital primordia, ISV = internal seminal vesicle, P = posterior, r = rostellum, s = suckers, SR = seminal receptacle, T = testes, U = uterus, VA = vagina, VOC = ventral osmoregulatory canal. Colorimetric whole-mount *in situ* hybridisation signal is shown in blue, FISH in green, DAPI in blue. Bars: 100 μm

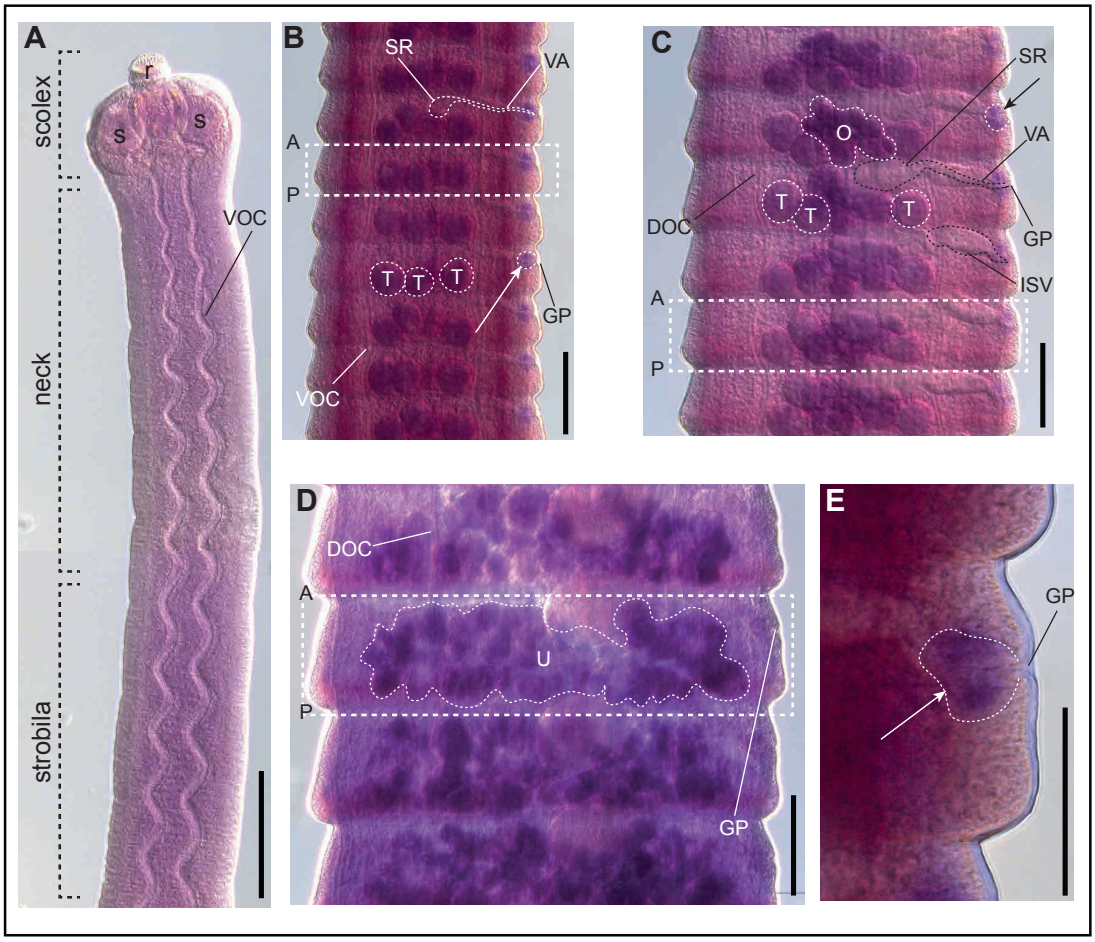

Figure 3.5. Expression of *Hmic-Notch2* in adult *Hymenolepis microstoma*. A) Expression in the adult worm is ubiquitous. In B) immature segments, and C) mature segments expression is stronger surrounding the genital pore (indicated by arrows). In C) gravid segments, expression is strongest within the uterus. E) Expression surrounding the genital pore in a mature segment (indicated by arrow). Dashed boxes designate one segment. A = anterior, DOC = dorsal osmoregulatory canal, GP = genital pore, ISV = internal seminal vesicle, O = ovary, P = posterior, r = rostellum, s = suckers, SR = seminal receptacle, T = testes, U = uterus, VA = vagina, VOC = ventral osmoregulatory canal. Bars: 100 µm

Within the base of the scolex, several foci of *Hmic-Ser* expression are found (Fig. 3.6 B). The position of this expression suggests that it is found at the location of major nerve junctions within the cephalic ganglia (Fig 1.4), further indicating an association with the nervous system. Weak, granular expression of *Hmic-Ser* is also observed along the midline of the neck that ceases once strobilation begins (Fig. 3.6 B), indicating potential involvement in early proglottisation.

RNA-Seq data shows *Hmic-Dl1* is upregulated in adults. Expression is ubiquitous across the adult worm (Fig. 3.7 A), although RNA-Seq data suggests increased expression in mature segments. Observation of *Hmic-Dl1* under high power indicates that there is consistent staining within the parenchyma (Fig. 3.7 E), explaining the pervasive blue staining seen across the entire worm. There is also stronger expression during development of the male system, in the early external seminal vesicle and testes (Fig. 3.7 B-C). In immature segments, three stronger foci of *Hmic-Dl1* appear, consistent with the developing testes (Fig. 3.7 C). Once segments have matured, single foci of expression remain within each testis (Fig. 3.7 D-E). Consistent, weak expression is also observed in embryos. Expression in mature testes and embryos may explain increased FPKM values in the end regions.

Hmic-Dl2 has a distinct pattern (Fig. 3.8 A). From the late neck, as strobilation begins, four distinct foci are seen – two dorsally and two ventrally (Fig. 3.8). Initially the foci appear to be located relatively centrally, but as segments become morphologically visible, the foci spread out, becoming

more laterally situated. The expression continues throughout adult development and the four foci are observed towards the posterior of every segment (Fig 3.7 B-D). The expression patterns of *Hmic-Dl2* are consistent with the location of the median nerve cords (Fig 1.4) and are most likely to coordinate with the major nerve junctions with the major transverse nerve along the posterior of each segment.

Hmic-Dl3 expression is extremely weak and appears punctate throughout the worm (Fig. 3.9 A-B). This punctate expression is limited to randomly distributed cells throughout the entire worm that appear to be located within both the cortex and medulla.

Like *Hmic-Dl3*, *Hmic-Dl4* is observed in granular expression that is strongest in the neck (Fig. 3.9 C). This granular expression continues and is observed more strongly in mature segments. This distribution of cells expressing *Hmic-Dl4* within each segment appears to be located within the cortex.

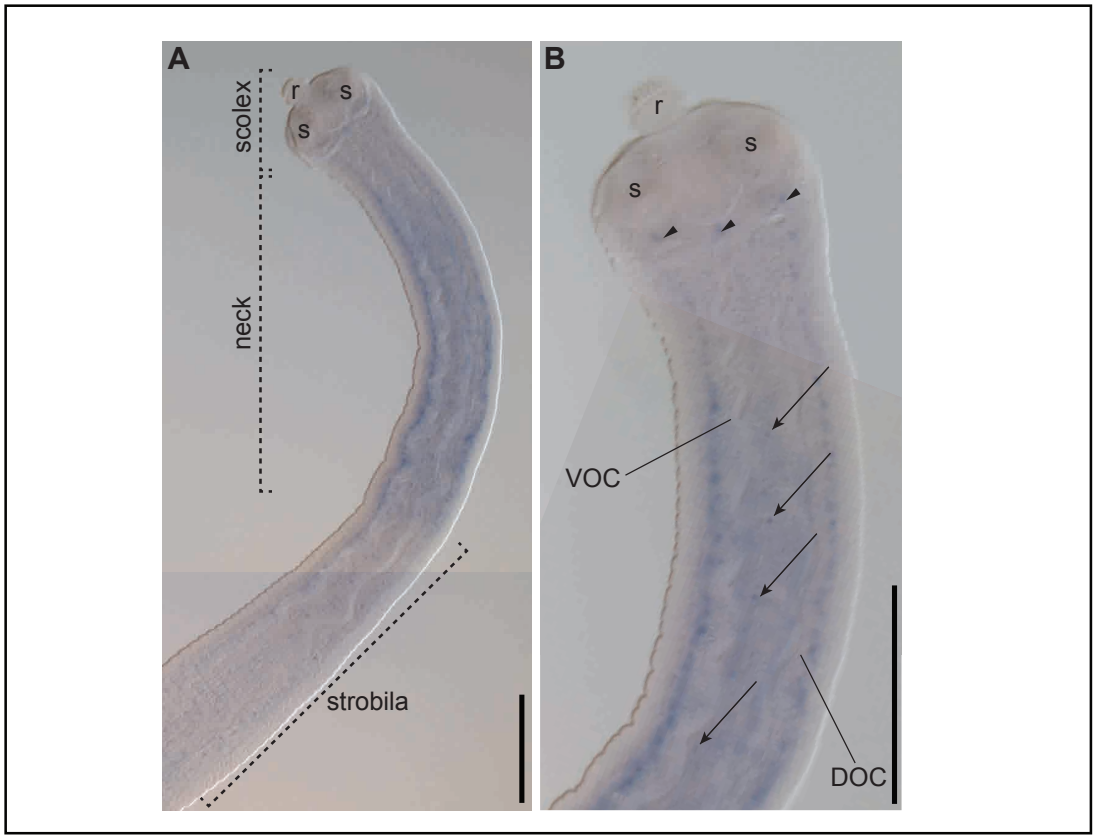

Figure 3.6. Expression of *Hmic-Ser* in adult *Hymenolepis microstoma*. Expression in A) the adult worm and B) scolex and neck. In the base of the scolex, discrete foci of expression are observed (arrowheads) that are likely to be within the cephalic ganglia. Expression is strongest in the neck where granular lateral stripes that follow the location of the lateral nerve cords. Weaker expression is observed along the midline (arrows). DOC = dorsal osmoregulatory canal, r = rostellum, s = suckers. Bars: 100 μm

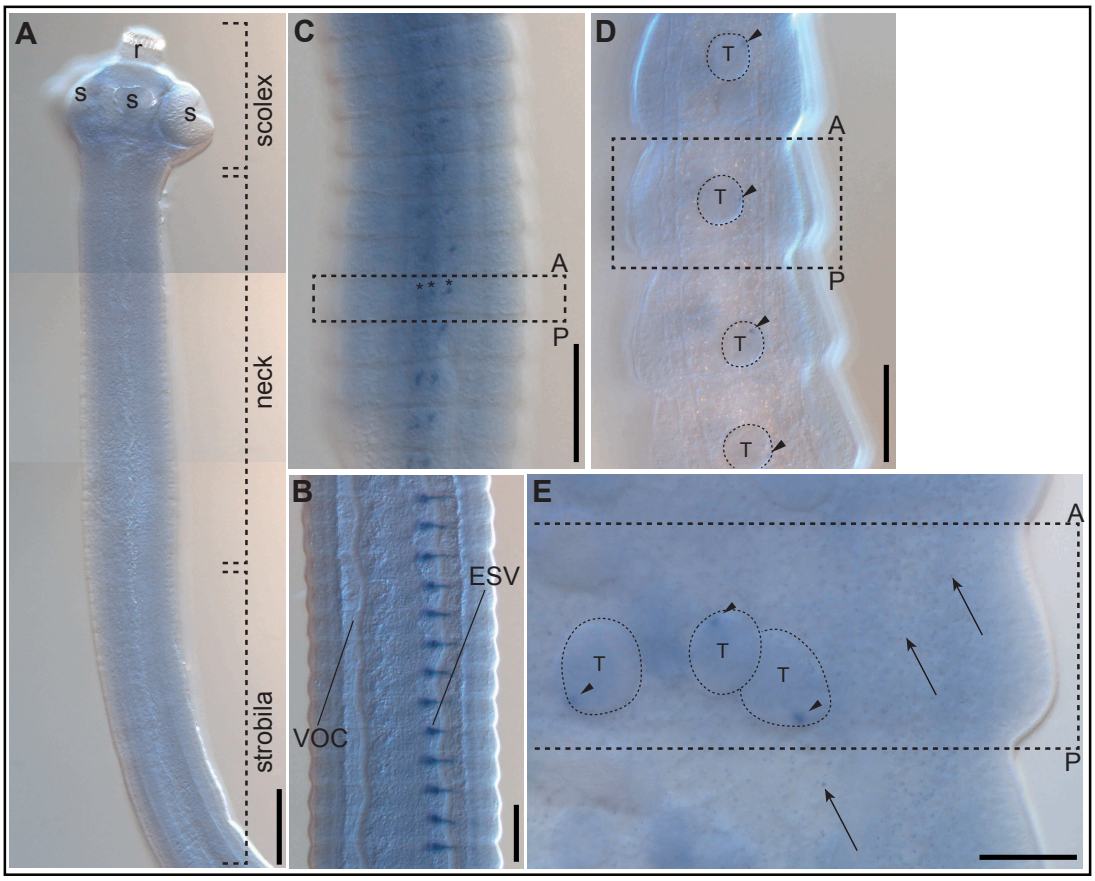

Figure 3.7 Expression of *Hmic-Dl1* in adult *Hymenolepis microstoma*. Expression in A) the adult worm is weak and ubiquitous. Expression within immature segments shows expression within the male reproductive system in B) the external seminal vesicle and C) developing testes. D) Dorsal view of a mature segment showing distinct foci within each testis (indicated by arrowhead). E) Discrete single foci are observed within the testes of mature segments (arrowheads). Ubiquitous granular staining within the parenchyma is observed (arrows). Dashed boxes designate one segment. A = anterior, ESV = external seminal vesicle, P = posterior, r = rostellum, s = suckers, T = testes, VOC = ventral osmoregulatory canal. Bars: 100 µm

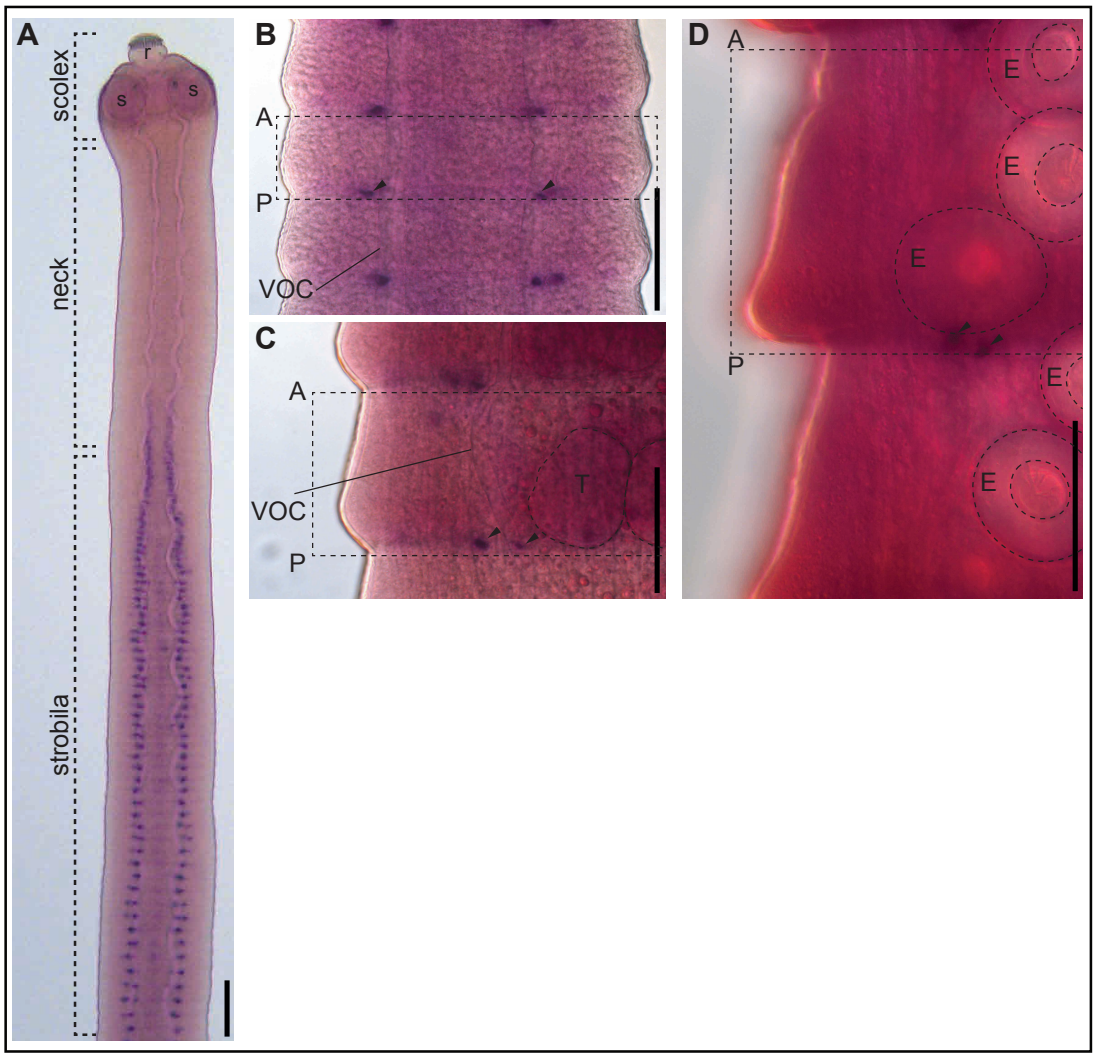

Figure 3.8 Expression of *Hmic-Dl2* in adult *Hymenolepis microstoma*. Expression in A) the adult worm shows *Hmic-Dl2* turns on towards the end of the neck and is observed as four foci, two ventrally and two dorsally that are expressed in the posterior of every segment. B) Once segments are fully established, the foci of *Hmic-Dl2* expression (arrowheads) are found at the posterior of each segment alongside the location of the median nerve cords. Expression is maintained in both C) mature segments and D) gravid segments. Dashed boxes designate one segment. A = anterior, E = eggs, P = posterior, r = rostellum, s = suckers, T = testes, VOC = ventral osmoregulatory canal. Bars: 100 µm

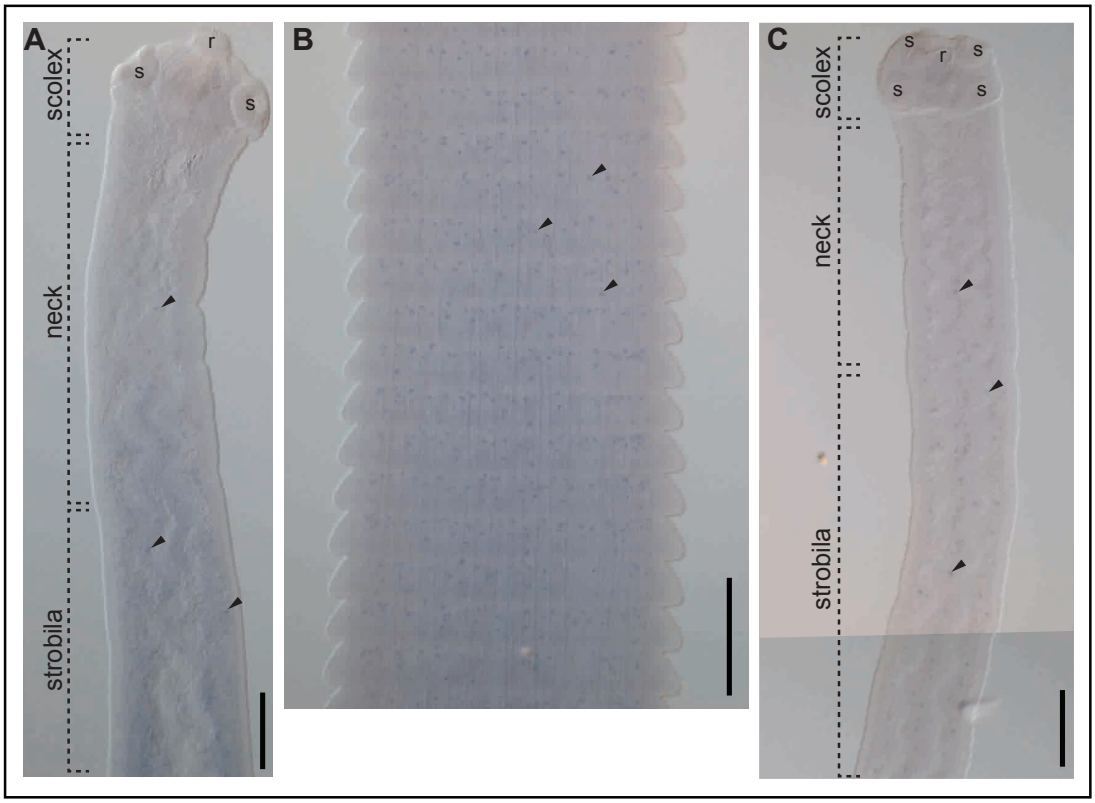

Figure 3.9 Expression of *Hmic-Dl3* and *Hmic-Dl4* in adult *Hymenolepis microstoma*. Expression of *Hmic-Dl3* in A) the scolex, neck and early strobila and B) immature segments. Expression is punctate (arrowheads) and is observed across the adult worm, although stronger in segments. C) *Hmic-Dl4* in the adult worm. Weak, granular expression (arrowheads) can be seen across the adult worm. r = rostellum, s = suckers. Bars: 100 μm

3.3 Discussion

3.3.1 Expression indicates multiple roles for Notch signalling in *Hymenolepis microstoma*

Notch signalling is extraordinarily pleiotropic and its outcomes are highly context-specific (Hori et al, 2013). This is echoed in the expression patterns of Notch factors in *H. microstoma*. In particular, *Hmic-Notch1* seems to play an important role across the entire development of *H. microstoma*, and is observed in several distinct expression patterns (Fig. 3.4). Expression in the mid-late neck is particularly of interest, as this is where new segments are being generated and the patterns observed here indicate a role for Notch signalling in several processes. When using FISH techniques that amplify the expression signal, ladder-like rings are is seen that as *Hmic-Notch1* expression matures, appear to be situated within the posterior of every segment (Fig. 3.4 B, E-F & I). This pattern strongly suggests that *Hmic-Notch1* is involved in the strobilation process of *H. microstoma*. A second potential role for Notch signalling in this region is found in the expression of longitudinal stripes in the neck (Fig. 3.4 A-D) that appear to mirror the location of the longitudinal and median nerve cords, and indicates a role in signalling within the nervous system. Finally, the central stripe (i.e. 'primitive streak') observed within the neck suggests a role in proglottisation and organogenesis. This is further supported by later expression of *Hmic-Notch1* within the seminal vesicle and ovary. Ubiquitous expression of *Hmic-Notch2* also indicates that Notch signalling is continuous across multiple cell types.

The wide expression of both *Notch* genes throughout adult development indicates that notch signalling is pleiotropic in *H. microstoma* and acts in a context-specific manner, a conserved feature with signalling in other systems (Louvi and Artavanis-Tsakonas, 2006; Kopan, 2012; Hori et al., 2013).

3.3.2 Notch signalling and strobilation

The striped ladder-like rings of *Hmic-Notch1* expression towards the end of the neck in newly forming segments and the later posteriorised expression within all segments strongly suggests a role for Notch signalling during strobilation. What is unclear is whether or not Notch is involved in establishing strobilation or if it is involved in the patterning of segments post strobilation. A well-known feature of Notch signalling is its role in segmentation, although it is not required for this process in all arthropods (Tautz, 2004; Peel et al., 2005). *Notch* itself is expressed in stripes along segment boundaries in several arthropod species, and in *P. americana*, knockdown of *Notch* can result in halting segmentation completely (Pueyo et al., 2008). In *G. bimaculatus*, however, knockdown of *Notch* does not prevent segment formation. Instead, it is involved in patterning segments post segmentation. (Kainz et al., 2011; Mito et al., 2011). Likewise, *Notch* is involved in the patterning of segments post segmentation in some annelids (Rivera and Wiseblat, 2009). The expression pattern of *Hmic-Notch1* indicates that the role of Notch signalling in segmentation is conserved in tapeworms (i.e. in strobilation). However, knockdown of *Hmic-Notch1* is still needed to establish if Notch signalling is involved in directing strobilation or is

only activated after the onset of strobilation, helping to pattern individual segments through specifying their polarity.

3.3.3 Notch signalling and the nervous system

The expression of two ligands and a receptor links Notch signalling with the nervous system. *Hmic-Dl2* is observed in foci that are consistent with the junctions between the medial nerve cords and the major transverse nerves. *Hmic-Ser* expression is found in nodes within the cephalic ganglia and lateral stripes consistent with the longitudinal nerves within the neck. *Hmic-Notch1* links the expression of *Hmic-Dl2* and *Hmic-Ser* as it is expressed in lateral and medial stripes in the neck. Further analysis is required to confirm co-localisation with neurons and would include co-staining with the neural markers synapsin or choline acetyltransferase (Nishimura et al., 2010).

It would also be interesting to examine the expression of the remaining ligands and downstream targets, which were not investigated, to explore further links with the nervous system. One of the roles Notch signalling plays in both vertebrates and invertebrates is that it acts to repress neurogenesis. Animals in which Notch signalling has been inhibited, excessive neurons are generated. Conversely, an increase in *Notch* activity results in a reduction of neurons. The expression patterns of *Hmic-Notch1*, *Hmic-Dl2* and *Hmic-Ser* in *H. microstoma* suggests that Notch signalling is also involved in the control of neuronal differentiation in tapeworms and identifies a putative

conservation of the neurogenic role of Notch signalling between tapeworms, other invertebrates and vertebrates.

3.3.4 Notch signalling and development of the reproductive system

Expression of *Hmic-Notch1* and *Delta1* is observed in developing female and male reproductive systems respectively. Notch signalling is widely involved during organogenesis and is observed during the development of gonads. Both oogenesis and spermatogenesis in *D. melanogaster* depend on Notch signalling (Xu et al., 1999; Lopez-Schier et al., 2001). During *D. melanogaster* oogenesis, Notch-mediated signalling controls the proliferation and differentiation of somatic cells (Lopez-Schier et al., 2001). The germline stem cell niche in the *D. melanogaster* ovary is regulated by Notch with knockdown of *Notch* resulting in a decrease in the production of cap cells (Song et al., 2007). Notch and its ligands are expressed in the mouse ovary and are required for the formation of follicles within the ovary (Trombly et al., 2009). The requirement of Notch signalling in ovarian development in *D. melanogaster* and mice and the expression of *Hmic-Notch1* in *H. microstoma* suggests that Notch signalling is required for correct development of the ovary in tapeworms.

Delta-Notch signalling specifies the cell fate of somatic and germ line stem cells in fly testes (Okegbe et al., 2011). The regulation of spermatogenesis is also coordinated by Notch signalling in mouse testes (Dirami et al., 2001; Tang et al., 2008; Murta et al., 2013). *Delta* is expressed in spermatagonia

and Sertoli cells in the mouse testis where Notch signalling was found to coordinate the proliferation and differentiation of cell fates during spermatogenesis (Dirami et al., 2001; Murta et al., 2013). Knockdown of Notch signalling results in the restriction of Leydig cell differentiation by promoting a progenitor cell fate instead (Tang et al., 2008). The role for Notch in regulating stem cell fate during spermatogenesis in both mice and flies may explain the foci of *Hmic-Dl1* expression in the testes of *H. microstoma*.

3.3.5 Notch signalling and embryogenesis

Hmic-Notch1 and *Hmic-Notch2* are both expressed in the uterus, suggesting that they are involved in the patterning of embryos. *Hmic-Notch1* can clearly be seen in individual cells of *H. microstoma* embryos *in utero*. Notch signalling is required to organise cell fate and patterning in the embryos during embryogenesis across the Metazoa. The expression of *Hmic-Notch1* and *Hmic-Notch2* during *H. microstoma* embryogenesis confirms that this is a conserved feature of Notch signalling in tapeworms.

3.3.6 Notch signalling and tapeworm germinative cells

Notch signalling is involved in the differentiation and proliferation of stem and progenitor cells in mice and *D. melanogaster*. This occurs in various stem cell populations including those associated with muscle, nerve and the somatic and germline cells of the ovary, testis. Recent models in planarians

show that neoblast subtypes and specialised progenitor cells exist alongside totipotent neoblasts (Zhu and Pearson, 2016). The expression of *Notch* receptors and their ligands indicates that Notch signalling may be involved in cell fate decisions through activation in localised, specialist germinative cell populations.

3.3.7 A role for Notch signalling in maintenance of muscle cells?

The expression of the ligands *Hmic-Dl3* and *Hmic-Dl4* suggests localisation in germinative cells. Germinative cells in tapeworms are found in higher concentrations within the medullary parenchyma, however, pulse chase-chase experiments show that these proliferative cells migrate into cortical regions (Koziol et al., 2010). Studies in planarians have shown the existence of specialised germinative cells (Zhu and Pearson, 2016). Although not confirmed, specialised germinative cells may also be present in other flatworms, including *H. microstoma*.

Notch signalling has been shown to mediate the restoration of muscle – insufficient activation of Notch signalling caused by a decrease in *Delta* with age leads to a reduction in muscle regeneration (Conbiy et al., 2003). Notch signalling is required to induce differentiation of stem cells into muscle (Kurpinski et al., 2010) and is required for the self-renewal and differentiation of muscle satellite cells (Bjornson et al., 2012). A reduction in *Notch* activity in quiescent satellite cells reduces the ability for muscle cells to be regenerated after injury (Bjornson et al., 2012). The localisation of *Hmic-Dl3*

and *Hmic-Dl4* coupled with their role in myogenesis suggests that could be expressed in specialised germinative cells involved in the maintenance of muscle cells in *H. microstoma*.

3.4 General conclusions and future directions

The presence of the majority of Notch signalling components in *H. microstoma* and other flatworms confirms that the Notch pathway is conserved among flatworms. The results of this chapter provide the first insights regarding Notch signalling in tapeworms and indicate that some features of Notch signalling are conserved amongst flatworms, arthropods and vertebrates, such as a potential role in the control of neurogenesis. The expression patterns of the Notch pathway factors examined indicate that Notch signalling plays diverse roles across the development of *H. microstoma*. Further exploration of the pathway in *H. microstoma*, including other paralogs of *Dl* and downstream targets such as the *Hes* family, was halted as early investigations suggested that Notch signalling does not play a role in tapeworm strobilation or polarity. However, more recent investigations looking at *Hmic-Notch1* expression using FISH (Fig. 3.4) suggests that Notch signalling does play a role in *H. microstoma* strobilation. Therefore, the role of Notch signalling in the segmentation process of other animals may be conserved with tapeworms. The more recent findings of *Hmic-Notch1* expression highlights further investigation of the Notch pathway in *H. microstoma* is still needed, especially in determining if it is also involved in establishing segment polarity in tapeworms.

Chapter 4

The Hedgehog pathway in *Hymenolepis microstoma*

4.1 Introduction

The Hedgehog (Hh) pathway (Fig. 4.1) is an evolutionarily conserved cell signalling system that is involved in the development of the Eumetazoa (Ingham et al., 2011). It controls several developmental processes including cell fate determination, growth and survival and a wide range of patterning processes. The ligand, Hh, acts as a morphogen and has the ability to act over both long and short ranges (Hooper, 2003; Varjosalo and Taipale, 2008). Hh is involved in several developmental events including the segmentation process, delineating developmental boundaries, guiding AP polarity, gut and neural formation. As a key factor in major patterning events, it is widely investigated and is of special interest as a causative factor in many human diseases – disruption of the Hh pathway is linked with tumorigenesis and growth of cancers (Taipale and Beachy, 2001; di Magliano and Hebrok, 2003).

4.1.2 *Hedgehog* discovery

The *Hh* gene was first discovered as a 'segment polarity gene' in *Drosophila melanogaster* due to the disruption of patterning and polarity of segments caused by its mutation (Nusslein-Volhard and Wieschaus, 1980). Mutation resulted in a duplication of denticles along the anterior of each segment and the loss of naked cuticle in the posterior. It is this 'continuous lawn' (Mohler, 1988) of spikey denticles, giving the appearance of a hedgehog-like ball, that gave the gene its name. *D. melanogaster Hh* was independently cloned by

four groups in the early 1990s who showed that the gene encoded a secreted protein (Lee et al., 1992; Mohler and Vani 1992; Tabata et al., 1992; Shigeki et al., 1993). Since its discovery in *D. melanogaster*, *Hh* has been identified in many animals from cnidarians to vertebrates (Ingham, Nakano, and Seger 2011). However, one notable exception is the lack of a recognisable *Hh* ortholog in *Caenorhabditis elegans*. Instead, *C. elegans* has several *hedgehog-related* genes (Aspöck et al., 1999). Surprisingly, *C. elegans* also lacks several other pathway components including *Costal2* (*Cos2*), *Fused* and *Suppressor of fused* (*Sufu*) or a recognisable *Smoothened* (*Smo*) gene (discussed below). Like many signalling systems, the pathway is best studied in *D. melanogaster*, although there are clear differences between *Drosophila* and vertebrate Hh signalling. One of the most notable differences is the duplication of pathway factors in vertebrates. Three paralagous hedgehog genes are present in mammals - *Sonic hedgehog*, *Indian hedgehog* and *Desert hedgehog* (Echelard et al., 1993) whilst zebrafish possess a further three paralogs (Ekker et al., 1995; Currie and Ingham, 1996; Ingham and McMahon, 2001). A second difference is the necessity (or lack thereof) of certain factors that are required for functional Hh signalling between different model systems (discussed below).

4.1.3 Mechanism of action

The mechanism of Hh signalling varies between *D. melanogaster* and vertebrates, though is still best understood in the fruit fly (Fig. 4.1). Signal-receiving cells have a 12-transmembrane pass receptor – patched (Ptc). In

uninduced cells, Ptc inhibits the action of a second transmembrane protein, Smo (an obligate transducer of the pathway), triggering its degredation and preventing its translocation to the cell membrane. In the absence of Hh signals, the protein Cubitus interruptus (Ci)/Gli, (a transcription factor that transduces Hh targets), is sequestered in the cytoplasm by Cos2 and Suppressor of fused (Sufu). Cos2 acts as a scaffold, and recruits several kinases: Fused, Casein kinase 1 (CK1), Glycogen synthase kinase 3 (GSK3) and Protein kinase 1 (PKA1) to form the Hh signalling complex (HSC). The kinases of the HSC promote the phosphorylation of Ci, generating recognition signals for the F-box protein, Supernumerary limbs (Slimb). Slimb then catalyses the ubiquitination of Ci into an N-terminal fragment that acts as a transcriptional repressor form of Ci (Ci-R). Ci-R dissociates from the HSC and moves to the nucleus where it represses transcription of Hh target genes, including *Ptc*, *Wg*/*Wnt* and *Decapentalplegic* (*Dpp*) (Alexandre et al., 1996).

The Hh protein has two distinct (and highly conserved) domains – the N-terminal 'Hedge' domain and the C-terminal 'Hog' domain. The Hog domain can be further split into two regions – an N-terminal 'Hint' domain and C-terminal sterol-recognition region (SSR) that binds cholesterol (Ingham et al., 2011). The Hog domain has ancient origins whilst the Hedge domain and Hh protein arose more recently (Bürglin 2008). It is likely that the Hh protein itself first arose in the common ancestor of the bilaterians and cnidarians (Richards and Degnan, 2009; Ingham et al., 2011). One possibility is that the

pathway arose from systems involved in the transport and homeostasis of lipids (Hausmann et al., 2009).

Full length Hh undergoes autoproteolysis in the endoplasmic reticulum that is promoted by the Hog domain and results in the release of Hedge and covalent coupling with cholesterol at the C-terminal (Briscoe and Therond, 2013). Before Hh-N can be released from the signalling cell it must undergo further modification by skinny hedgehog (ski)/rasp that catalyses palmitoylation of Hh-N. The multipass transmembrane protein Dispatched (Disp) is required for the release of Hh. It acts as a maturase, forming multimeric complexes of Hh that are thought to be necessary for the movement of Hh through tissues (Burke et al., 1999; Kawakami et al., 2002; Ma et al., 2002). In Disp mutants, Hh accumulates in Hh producing cells and the resulting transcription of target genes under normal conditions is lost. Several other genes have been indicated in the movement of Hh, aiding in its secretion and reception. These include the glypicans *Dally* and *Dally-like* (*Dlp*) genes that act as receptors and encode heparan sulphate proteoglycans (Desbordes and Sanson, 2003) that are also required for regulation of Wnt and Dpp signalling (Tsuda et al., 1999). The synthesis of these proteoglycans requires glycosyltransferases that are encoded by the exostosin-related genes *Tout velu* (*Ttv*), *Sister of tout velu* (*Sotv*) and *Brother of tout velu* (*Botv*) (Bornemann et al., 2004; Takei et al., 2004).

Induction of the Hh pathway occurs through receptor inactivation – binding of Hh to Ptc inhibits the function of Ptc, leading to pathway activation. When Hh

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Figure 4.1 The Hh signalling pathway. A) Hh signalling in *Drosophila*. B) Hh signalling in vertebrates. See image text and chapter for a more detailed description of the pathway's mechanism of action and factor interactions. (Adapted from Robbins et al., 2012)

is released from a signalling cell, it binds to Ptc which is expressed on the surface of a receiving cell. Pathway activation can be mediated by the co-receptors Interference hedgehog (Ihog)/Cdo and Brother of interference hedgehog (Boi)/Boc (Beachy et al., 2010; Allen et al., 2011). In *D. melanogaster*, Ptc can bind Hh independently of Ihog and Boi, however their simultaneous inactivation results in inactivation of the pathway (Beachy et al., 2010). In the absence of Hh, Ptc suppresses the activity of Smo.

Conversely, when Hh is bound to Ptc, Smo is activated. Smo is an obligate activator of Hh signalling – its inactivation prevents transcription of pathway target genes (Ingham and McMahon, 2001). Smo accumulates and is phosphorylated by PKA, CK1, GSK3, leading to its dimerization and translocation to the cell membrane (Su et al., 2011). Phosphorylation of the protein leads to a conformational change in the cytoplasmic tail of Smo that recruits the binding of Cos2/Kif7 and Fused and their dimerisation. This activates Fused, an essential component of Hh signalling (its loss prevents activation of target genes (Zhou and Kalderon, 2011)) that phosphorylates Cos2/Kif7 and Sufu (Shi et al., 2011). Phosphorylation of the Cos2/Kif7-Fused complex causes the release of full-length Ci from the HSC and Ci levels subsequently increase in the cytoplasm. Full length Ci moves into the nucleus where it is modified further into the activated form (Ci-A). Ci-A then interacts with the co-activator CREB-binding protein (CBP) and the transcription of target genes, such as Wnt and Dpp (Alexandre et al., 1996), is initiated. Interestingly, several targets of Hh signalling are components of

the pathway, including *Ptc* and *Ci*/*Gli*, creating self-regulating, negative and positive feedback loops, respectively.

Many components of Hh signalling are shared between *Drosophila* and vertebrate signalling, as are the mechanisms of how they interact, however there are some differences between the two systems. 1) Duplications of multiple factors (including *Hh*, *Ptc* and the vertebrate homolog of *Ci – Gli*) have occurred in vertebrates (Lee et al., 2016). 2) *Sufu* is indispensable in vertebrate Hh signalling and its loss leads to embryonic lethality in mice (Svärd et al., 2006). 3) Vertebrate Hh signalling is dependent on primary cilia, with key factors expressed within cilia, that may act as antennae to facilitate signalling (reviewed in Eggenschwiler and Anderson, 2007; Wilson and Stainier, 2010; Lee et al., 2016). The association of cilia with Hh signalling has also been implicated in Planaria – the pathway is required for planarian ciliogenesis, suggesting that planarians, like vertebrates, rely on cilia for functional Hh signalling (Rink et al., 2009).

4.1.4 Hedgehog and segmentation

A cascade of genes controls segmentation in the *D. melanogaster* embryo (reviewed in Hartenstein and Chipman, 2015). The final genes to be activated in this cascade are the 'segment polarity genes' that include *Engrailed*, *Hh* and *Wg*/*Wnt* that act as part of a segment polarity network (von Dassow et al., 2000). *Hh* and *Wg*/*Wnt* are expressed in unique domains that border each other and define the parasegmental boundary that is critical

for maintaining borders. Loss of *Hh* results in abnormal expression of *Wg*/*Wnt* and duplication of segment boundaries. *Hh* helps to further pattern the AP axis of each segment (in concert with *Wg*/*Wnt*) through regulation of Notch and EGFR signalling (Alexandre et al., 1999). These findings are echoed in the flour beetle, *Tribolium castaneum*, in which patterns of *Hh, Ci*, *Ptc* and *Smo* expression are conserved with *D*. *melanogaster*. RNAi of *Hh*, *Ci* and *Smo* result in the loss of segment maintenance, and confirms a conserved role of Hh signalling as part of a *En*-*Hh*-*Wg* circuit in the patterning and maintenance of segments (Farzana and Brown, 2008).

Hh signalling also controls maintenance and patterning of segments in non-insect arthropods. *Hh* seems to act as a segment polarity gene in both the scorpion, *Euscorpius flavicaudis*, and the crustacean, *Artemia franciscana* (Simonnet et al., 2004). *Hh* is observed in stripes prior to morphological segmentation in both organisms (in the posterior growth zone – *E*. *flavicaudis* and in the thorax – *A*. *franciscana*). Once segments become morphologically visible, *Hh* expression is seen along the posterior border of segments (Simonnet et al., 2004). In the sister group to Arthropoda, Onychophora, a conserved role of Hedgehog signalling in defining segment boundaries is not observed. Expression of *Hh* in *Euperipatoides kanangrensis* does occur in stripes along segment borders, but only after segmentation, indicating that is not involved in the segmentation process. Although, the activation of *Hh* by *En* and subsequent transcription of *Wg/Wnt* does seem to be conserved between onychophorans and arthropods (Janssen and Budd, 2013).

In the polychaete annelid, *Platynereis dumerilii*, Hh signalling is observed to regulate segment formation. *Hh*, *Ptc* and *Ci*/*Gli* are all expressed in stripes before the morphological appearance of segmentation in both larvae and during posterior growth of adults (Dray et al., 2010). However, chemical inhibition of Hh signalling with cyclopamine indicates that it is not required for the formation of segments, as the segment polarity genes *Engrailed*, *Nk4* and *Lbx* are still expressed in stripes during early larval development. Disruption during mid-larval development, however, results in the loss of the striped expression of *Wnt1*, *Hh* and *Engrailed*, suggesting that Hh signalling is required to maintain segmental patterns prior to the onset of visible, morphological segments (Dray et al., 2010). In the closely related *Perinereis nuntia*, *Hh* expression appears cyclically in stripes and is thought to act in the segmentation process in association with *Wnt* (Niwa et al., 2013).

Studies in other annelids have, however, questioned a conserved role for Hedgehog signalling in segment formation and maintenance. Hh signalling in the leech, *Helobdella robusta*, is instead involved in formation of the gut and neural differentiation. Inhibition of the pathway using cyclopamine disrupts gut formation (Kang et al., 2003). Likewise, a role in patterning of the gut and specification of the hindgut is observed in *Capitella* sp. I (Seaver and Kaneshige, 2006). Expression of *Capitella Hh* indicates that it is not involved in the patterning of annelid segments and is instead found in a number of tissues including the gut and nervous system.

4.1.5 Hedgehog signalling in AP polarity and neural maintenance in planarians

In planarians *Hh* is expressed in the brain, along the ventral nerve cords (VNCs), eyes and anterior tip of the head (Rink et al., 2009; Yazawa et al., 2009). Other factors (*Ptc*, *Gli* and *Sufu*) are expressed more ubiquitously (Yazawa et al., 2009) although *Ptc* expression is stronger within the brain and along the VNCs, as is *Smo* (Rink et al., 2009). Knockdown of Hh signalling by RNAi of *Ptc* causes regenerating planarians to become 'headless' and, in increased dosages, to grow an anterior tail rather than a head (Rink et al., 2009; Yazawa et al., 2009; Glazer et al., 2010).

Conversely, *Hh* RNAi during regeneration leads to 'tailless' animals and even the formation of a posterior head. Furthermore, Hh signalling has a clear role in the establishment of planarian AP polarity by regulating *Wnt* expression during regeneration (Rink et al., 2009; Yazawa et al., 2009). These studies identified that *Hh*, *Gli* and *Smo* promote tail decisions by stimulating *Wnt1* expression whilst *Ptc* and *Sufu* promote head decisions through *Wnt1* inhibition. Yazawa et al. (2009) proposed a model linked with the observed expression of *Hh* within the nervous system. In this model, *Hh* gene products are transported posteriorly along the VNCs, creating a gradient that directs head or tail regeneration according to the AP polarity of the injured fragment.

More recently, the potential role of Hh signalling in the maintenance and/or regeneration of the planarian nervous system has been investigated further.

The expression of genes in glial cells is controlled by proximity to neurons positive for *Hh* (Wang et al., 2016). Building on this, the main source of planarian Hh signals are the ventral medial neurons (Currie et al., 2016). Co-localisation with the stem cell marker *Piwi* identified that neoblasts near the brain express core Hh pathway transduction genes – *Ptc*, *Smo* and *Gli*, suggesting that neoblasts adjacent to the nervous system are the targets of Hh signalling (Currie et al., 2016). Additionally, a reduction in Hh signalling led to a decrease in the production of neural progenitor cells and new cholinergic neurons – consistent levels of Hh signalling is required to maintain normal production and turnover of these cells, however, the mechanism by which this occurs is still unclear (Currie et al., 2016).

Hh signalling clearly fulfils many different roles in different organisms. The close phylogenetic relationship between tapeworms and planarians would suggest that Hh signalling is likely to be involved in guiding the AP polarity of *H. microstoma* and possibly neurogenesis. Coupled with this, a role for Hh signalling in the segmentation of annelids and arthropods could also suggest a potential role in the strobilation process of tapeworms. Here the presence of Hh pathway factors and their spatial expression patterns in *H. microstoma* were examined.

4.2 Results

4.2.1 Genomic analysis

Genomic analysis shows that the Hh pathway is widely conserved in *H. microstoma*. BLAST searches identified single copies of the 'core' Hh pathway components *Hh*, *Ci*, *Ptc*, *Smo*, *Fused*, *Sufu*, *Disp* and *Slimb* (Table 4.1). The one notable exception is the absence of a *Cos2*/*Kif7* ortholog in *H. microstoma*. Multiple copies of *Rsp*, *PKA*, *CK1*, *GSK3* and *CBP* were present in the genome whilst all other factors identified had a single copy (Table 4.1).

Analysis of predicted domain architecture of the predicted Hh protein confirmed it as a true Hh protein containing a Hedge and Hog domain (Fig. 4.1). Phylogenetic analysis with other *Hh* genes follow the same patterns seen in current lophotrochozoan relationships (data not shown). The single *Hh* in *H. microstoma* indicates that no gene expansion of *Hh* has occurred within tapeworms. A single copy in two planarians (Rink et al., 2009; Yazawa et al., 2009) as well as gene mining in other flatworms further suggests that this is true across the Platyhelminthes. This pattern holds true with only one homolog of *Ptc*, *Smo*, *Disp*, *Fused*, *Sufu* and *Slimb* present in *H. microstoma* (and other flatworms) and their predicted protein structures all possess their characteristic diagnostic domains (Fig. 4.2 and Table 4.1). One copy of *Ci* is present in the *H. microstoma* genome. This echoes the finding observed in *Dugesia japonica* (Yazawa et al., 2009) but contrasts with the three homologs found in *S. mediterranea* (Rink et al., 2009). Phylogenetic analysis

suggests that this expansion has occurred via duplication in *S*. *mediterranea* rather that a loss in other flatworms (data not shown).

Surprisingly, no homolog of *Cos2*/*Kif7* could be found. This is highly unusual given the importance of this gene and its requirement for Hh signalling in sequestering Ci and acting as a scaffold in the HSC (Jia et al., 2003; Zhang et al., 2005; Liem et al., 2009). Multiple *Kif* genes were identified in *H*. *microstoma* through BLAST analysis (data not shown), but none were identified as *Cos2*/*Kif7*. *Kif27* was identified as the most likely homolog of *Cos2/Kif7* in *S*. *mediterranea* (Rink et al., 2009). BLAST searches against *H*. *microstoma* gene models with *Smed-Kif27* resulted in three possible *H*. *microstoma* homologs. However, reciprocal BLAST searches against the *S*. *mediterranea* genome resulted in the top hit for each of these three genes to be to *Smed-Kif3*. The third hit was to *Smed-Kif27* however BLAST searches against the *D*. *melanogaster*, mouse and human genomes gave *Kif17*, *Kif11* and *Kif1* as the top hits for each gene respectively. This discrepancy and apparent lack of a *Cos2*/*Kif7*/*Kif27* homolog in *H*. *microstoma* is most likely due to high sequence divergence of this gene rather than its loss from the genome.

No orthologs of the co-receptors *Dally*, *Dally-like*, *Ihog*, *Boi*, *HHIP*, or the regulators *Talpid3* and *Rab23* could be identified. Absence of *HHIP* is consistent with previous findings of the gene as a vertebrate-specific inhibitor of Hh signalling (Bishop et al., 2009). The absence of the co-receptor *Dally*, *dlp*, *Ihog* and *Boi* indicates that they have been lost from the *H*. *microstoma*

Table 4.1 Hedgehog pathway factors in *H. microstoma*. *H. microstoma* Hedgehog pathway factors, their predicted gene model numbers and the length of predicted proteins in base pairs. RNA-Seq data for each gene model is given in the number of reads per kilobase per million mapped reads (RPKM) for four stages of development – mid larval stages, whole adult, the scolex/neck, mid (i.e. mature segments) and end (i.e. gravid segments). Differential expression between regions is given (from Tsai et al., 2013).

Gene	Gene model	Length (bp)	RPKM values					Differential expression			
			Larva	Whole adult	Scolex/ neck	Mid regions	End regions	Larva vs whole adult	Scolex/neck vs mid	Scolex/neck vs end	Mid vs end
Hmic-Hh	HmN_000068600	1584	51.1	16.2	31.1	10.9	8.5	ND	ND	DOWN	ND
Hmic-Ci	HmN_000840400	4839	50.6	49.0	20.3	21.7	9.4	ND	ND	DOWN	ND
Hmic-Disp	HmN_000570400	3120	30.9	71.5	15.0	76.9	16.4	ND	UP	ND	DOWN
Hmic-Ptc	HmN_000602000	4467	94.3	62.5	30.8	24.4	20.0	ND	ND	ND	ND
Hmic-Fu	HmN_000686000	4473	15.6	39.7	11.8	18.9	14.0	ND	ND	ND	ND
Hmic-Sufu	HmN_000482400	2640	62.4	72.7	35.0	39.2	23.4	ND	ND	ND	ND
Hmic-Smo	HmN_000930900	4581	29.1	33.4	10.1	18.9	11.6	ND	ND	ND	ND
Hmic-Slmb	HmN_000834500	2235	99.9	143.5	55.9	93.9	65.1	ND	ND	ND	ND
Hmic-PKA1	HmN_000251300	1053	109.0	246.1	268.5	142.3	217.7	ND	ND	ND	ND
Hmic-PKA2	HmN_000254800	1203	16.4	156.2	115.4	70.4	43.4	UP	ND	DOWN	ND
Hmic-CK1-1	HmN_000253700	1128	66.9	536.4	191.2	230.4	336.5	ND	ND	UP	ND
Hmic-CK1-2	HmN_000690200	921	10.6	131.6	32.4	103.2	162.0	UP	UP	UP	ND
Hmic-CK1-3	HmN_000690500	900	66.1	114.0	111.9	78.7	95.8	ND	ND	ND	ND
Hmic-CK1-4	HmN_000752200	1053	0.0	147.0	1.9	141.2	15.8	ND	UP	UP	DOWN
Hmic-CK1-5	HmN_000297300	1410	74.3	84.7	43.7	55.9	51.7	ND	ND	ND	ND
Hmic-CK1-6	HmN_000348900	1128	66.9	536.4	191.2	230.4	336.5	ND	ND	UP	ND
Hmic-GSK3β1	HmN_000354400	1227	84.5	154.6	76.5	102.3	135.4	ND	ND	UP	ND
Hmic-GSK3β2	HmN_000472700	1431	228.8	184.7	114.5	133.5	102.1	ND	ND	ND	ND
Hmic-CBP1	HmN_000030800	498	0.1	29.5	0.7	64.7	4.6	UP	UP	ND	DOWN
Hmic-CBP2	HmN_000269800	5583	65.8	87.6	16.9	31.0	16.5	ND	ND	ND	ND
Hmic-CBP3	HmN_000466200	645	0.0	21.1	0.2	25.6	3.0	ND	ND	ND	DOWN
Hmic-CBP4	HmN_000479000	5328	18.8	31.9	5.6	12.9	13.7	ND	ND	UP	ND
Hmic-CBP5	HmN_000756700	597	0.0	41.4	1.9	112.8	7.8	UP	UP	ND	DOWN
Hmic-CBP6	HmN_000467600	3468	0.7	31.1	0.1	22.8	10.2	UP	UP	UP	ND
Hmic-CBP7	HmN_000466400	1905	0.0	77.4	0.7	152.1	22.1	ND	UP	UP	DOWN
Hmic-Rsp1	HmN_000543900	1098	14.7	1.8	6.3	1.3	0.4	DOWN	ND	DOWN	ND
Hmic-Rsp2	HmN_000846200	1419	1.1	2.5	0.4	1.1	2.4	ND	ND	ND	ND
Hmic-Rsp3	HmN_000846300	1431	28.6	33.9	21.2	26.5	44.8	ND	ND	UP	ND
Hmic-Igu	HmN_000604900	2622	5.0	13.5	3.0	9.4	2.8	ND	UP	ND	ND
Hmic-Rdx	HmN_000637800	2247	60.4	121.2	46.2	98.0	68.9	ND	UP	ND	ND
Hmic-Ttv	HmN_000215200	2325	56.6	33.7	27.5	27.2	29.5	ND	ND	ND	ND
Hmic-Sotv	HmN_000928900	1713	109.2	54.6	51.2	45.8	27.3	ND	ND	DOWN	ND
Hmic-Botv	HmN_001006500	2259	8.4	43.2	22.8	25.8	51.3	UP	ND	UP	ND

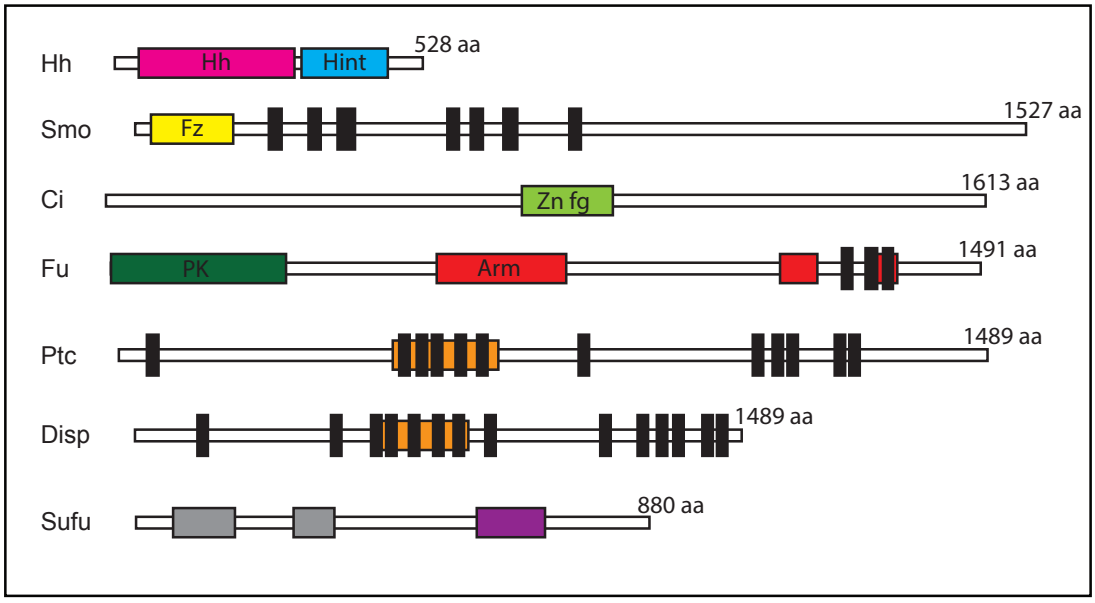

Figure 4.2 Structure of Hedgehog pathway factors in *Hymenolepis microstoma*. The domain organisation of proteins belonging to the Hh pathway. Smart and InterPro were used for domain analysis and the major domains used to define the proteins are shown. The number of amino acids in each protein is also given. Diagnostic domains: Arm/red = armadillo-type fold, Hh = hedgehog domain, Hint = intein domain, Fz = frizzled domain, PK = protein kinase domain, Zn fg = zinc finger, orange = sterol-sensing domain, grey = suppressor of fused-like domain, purple = suppressor of fused C-terminal, black = transmembrane region.

genome as they are present in the cnidarian, *Nematostella vectensis* (Matus et al., 2008). This suggests that these co-receptors are not required for effective Hh signalling in *H. microstoma*. This echoes findings in *D. melanogaster*, in which Hh signalling can act independently of Ihog and Boi, although simultaneous knock down impairs Hh signalling (Beachy et al., 2010).

4.2.2 RNAseq analysis

Alongside the *H. microstoma* genome, transcriptomes of various stages of development (larvae, whole adults and three regions of the adult worm) were produced (Tsai et al., 2013). RNAseq data of the genes identified through analysis of the genome as belonging to the Hh pathway can be seen in Table 4.1. Looking at these data, 21 genes belonging to the Hh pathway have statistical levels of differential expression between either larval and adult stages or between any of the three regions of the adult worm (Table 4.1). Three of these can be classified as 'core' factors – *Hmic-Hh*, *Hmic-Ci* and *Hmic-Disp*. *Hmic-Hh* expression is shown to be stronger in the neck – here it is almost three times stronger than in the mid or end regions of the strobila (Table 4.1). The stronger expression of *Hmic-Hh* in the scolex/neck that decreases after the event of strobilation suggests a possible role for *Hmic-Hh* in either (or both) the strobilation or proglottisation process. *Hmic-Ci* levels are fairly consistent, although it is expressed at lower levels towards the end of the strobila, halving in end regions (Table 4.1). *Hmic-Disp*, that encodes a protein necessary for the transportation of Hh is upregulated in

immature segments (Table 4.1), suggesting that Hh signalling may be required for the development and maturation of tapeworm gonads.

The remaining factors showing differential expression between different stages and regions are modifiers and regulators of the Hh pathway. They are; *Hmic-Rsp*, *Hmic-PKA*, *Hmic-CK1*, *Hmic-CBP*, *Hmic-Iguana*, *Hmic-Rdx*, *Hmic-Sotv* and *Hmic-Botv*. As a general rule, many of these genes show increased expression in adults compared with larvae and in particular, increased expression within the mid-regions of the adult worm, i.e. immature segments (Table 4.1). This suggests a possible role in gonad development. However, several of these differentially expressed genes are involved in other signalling pathways, such as Wnt and FGF signalling (e.g. Chen et al., 2000; Bornemann et al., 2004; Han et al., 2004; Takei et al., 2004; Price 2006; Wu and Pan, 2010; Fischer et al., 2011) and so may not be directly involved with Hh signalling.

4.2.3 Expression of Hedgehog pathway factors in larvae

WMISH of *Hmic-Hh* in early larvae shows two stripes that are located along the dorsoventral axis running down from the anterior apex (Fig. 4.3 A and 5.18 C). As the larvae mature, these stripes change into patches of clustered expression that remain along the dorsoventral axis. By the time larvae have developed into cysticercoids, expression of *Hmic-Hh* is in discrete nodes that run along the midline of the newly developed scolex (Fig. 4.3 A).

The expression of *Hmic-Ci* in *H. microstoma* larvae is generally posteriorised (Fig. 4.3 B). During early stages of development, *Hmic-Ci* is observed in a few cells towards the posterior pole and surrounding the primary lacuna (Fig. 4.3 B). As the larvae elongate, reaching Stage III, expression only occurs in the posterior of larvae (Fig. 4.3 B). Once fully developed, the expression is restricted to the cyst tissue surrounding the juvenile worm (Fig. 4.3 B).

The expression patterns of *Hmic-Ptc* and *Hmic-Smo* are similar (Fig. 4.3 C and 4.4 B). During Stage I-II, expression of both genes is weak, and generally posteriorised, with a few scattered anterior cells expressing *Hmic-Ptc* and *Hmic-Smo* (Fig. 4.3 C and 4.4 B). Once the larvae have reached Stage III, expression is only observed in the posterior and this continues as larvae develop into Stage V, with *Hmic-Ptc* and *Hmic-Smo* expression only found in the posterior of the cyst tissue (Fig. 4.3 C and 4.4 B).

Ubiquitous expression of *Hmic-Disp* during early development is observed, although expression is marginally stronger towards the posterior (Fig. 4.4 A). As larvae elongate, expression of *Hmic-Disp* becomes more posteriorised, and is observed from the midline to the posterior pole in tissues surrounding the primary lacuna (Fig. 4.4 A). Once fully developed, *Hmic-Disp* is seen in the posterior and extending to the midline of the cyst tissue (Fig. 4.4 A).

The expression of *Hmic-Slimb* is weak during early-mid larval development (Fig. 4.4 C). During Stage I, this weak expression is ubiquitous, but by Stage III is restricted to the posterior half of larvae (Fig. 4.4 C). As with many other

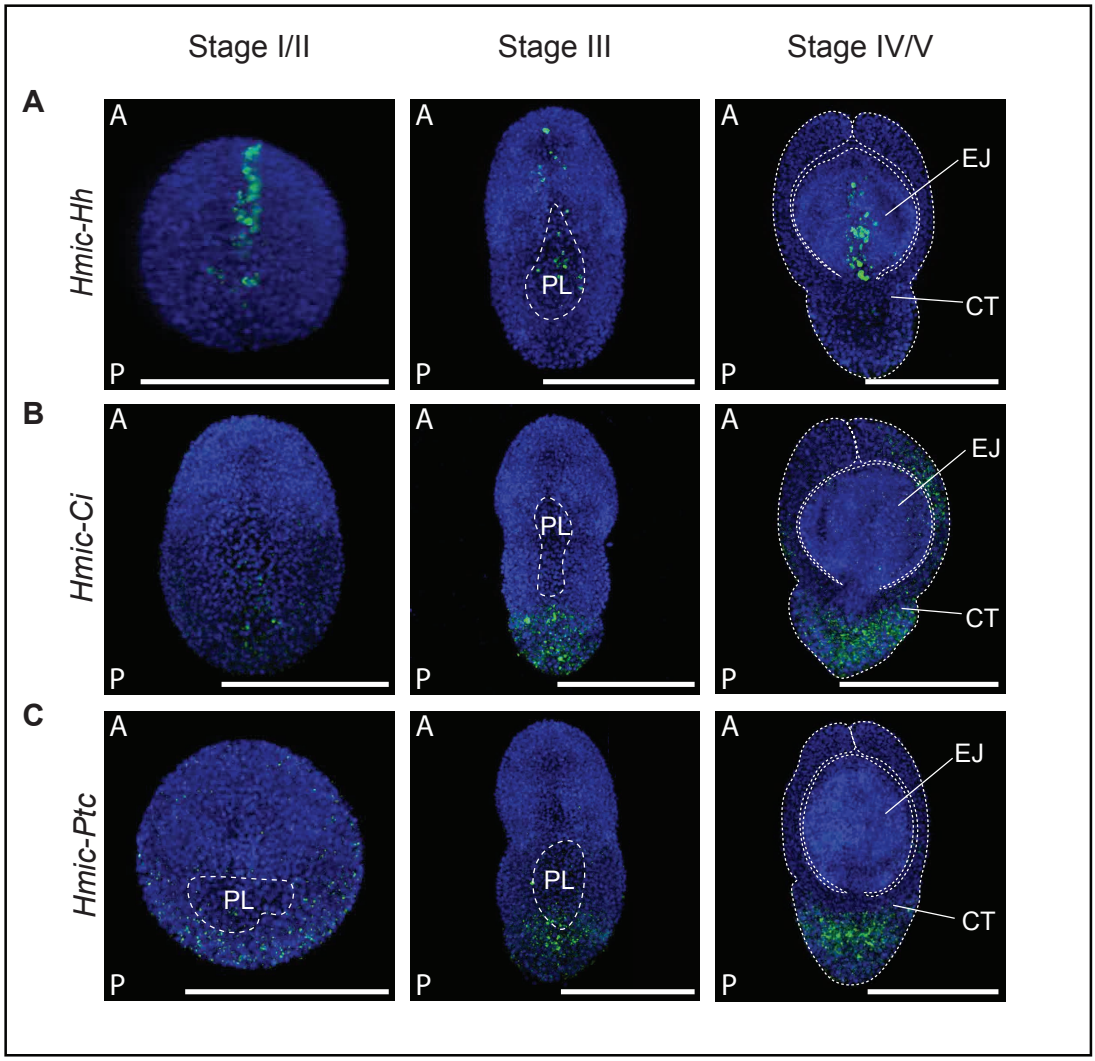

Figure 4.3 Expression of *Hmic-Hh*, *Hmic-Ci* and *Hmic-Ptc* during *Hymenolepis microstoma* larval development. FISH maximum projections of A) *Hmic-Hh*, B) *Hmic-Ci* and C) *Hmic-Ptc*. Larvae are staged according to Voge (1964). A = anterior pole, CT = cyst tissue, EJ = encysted juvenile worm, h = hooks, P = posterior pole, PL = primary lacuna. Whole-mount *in situ* hybridisation signal is shown in green and DAPI in blue. Bars: 50μm

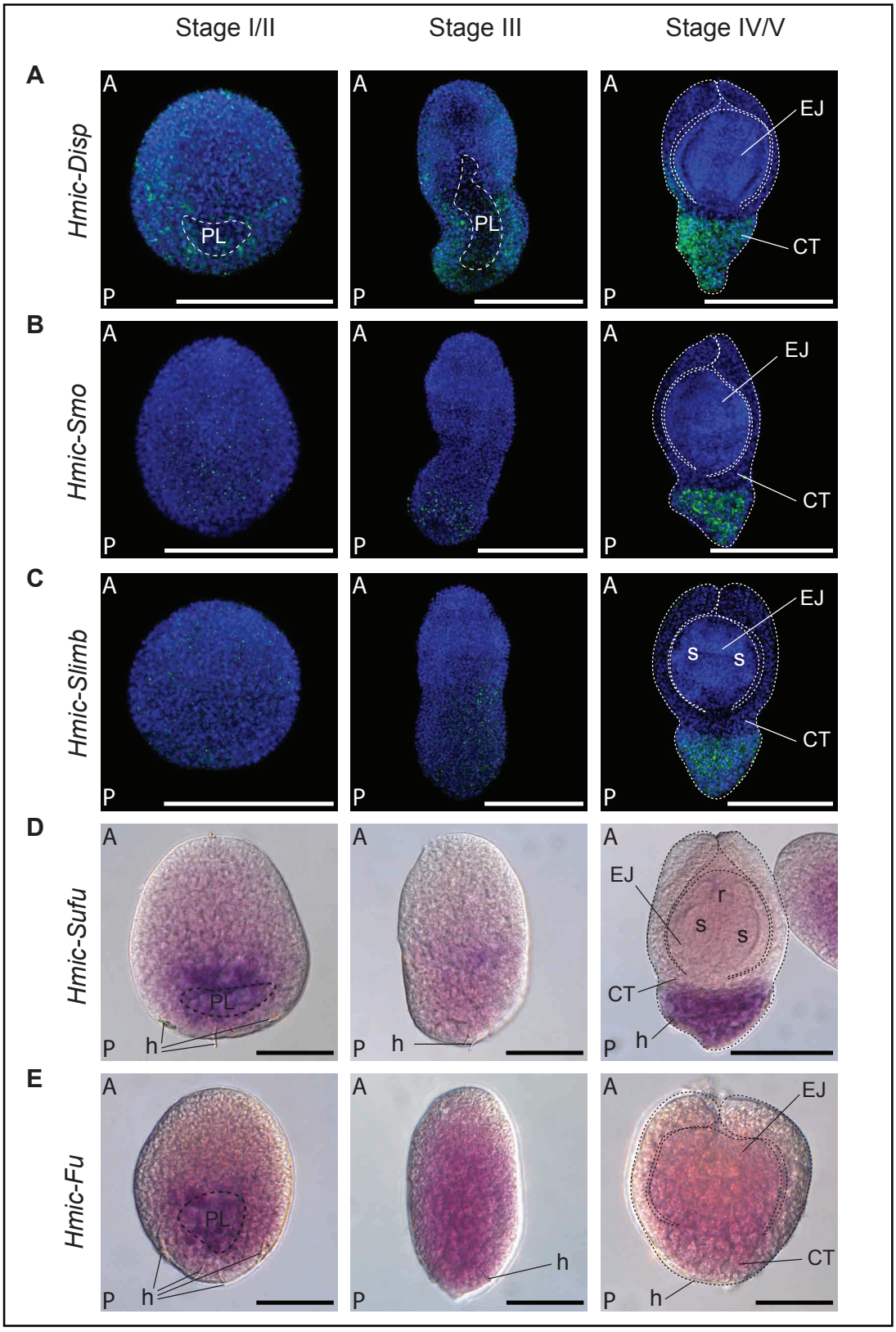

Figure 4.4 Expression of *Hmic-Disp*, *Hmic-Smo*, *Hmic-Slimb*, *Hmic-Sufu* and *Hmic-Fu* during *Hymenolepis microstoma* larval development. FISH maximum projections of A) *Hmic-Disp*, B) *Hmic-Smo* and C) *Hmic-Slimb*. Colorimetric ISH of D) *Hmic-Sufu* and E) *Hmic-Fu*. Larvae are staged

according to Voge (1964). A = anterior pole, CT = cyst tissue, EJ = encysted juvenile worm, h = hooks, P = posterior pole, PL = primary lacuna, r = rostellum, s = suckers. Colorimetric whole-mount *in situ* hybridisation signal is shown in purple, FISH in green and DAPI in blue. Bars: 50μm

genes studied in larvae, *Hmic-Slimb* expression by stage V is only found in the posterior of the cyst tissue (Fig. 4.4 C).

WMISH of *Hmic-Sufu* identifies that expression is restricted to the posterior half of larvae throughout development (Fig. 4.4 D). During early stages, expression is strongest towards the posterior, whilst by Stage III, expression is observed from the mid line to the posterior pole (Fig. 4.4 D). Once *H. microstoma* larvae are fully infective at Stage V, expression of *Hmic-Sufu* is restricted to the posterior of the cyst tissue (Fig. 4.4 D).

Expression of *Hmic-Fu* is much more ubiquitous (Fig. 4.4 E) than any other genes expressed during larval development. At Stage I, *Hmic-Fu* appears to be stronger towards the posterior, however during mid-late development, no distinct patterns can be observed, with expression occurring through all larval tissues (Fig. 4.4 E).

4.2.4 Expression of Hedgehog factors in adults

The expression of *Hmic-Hh* in adult worms is striking (Fig. 4.5 A-B). Foci are observed within the scolex towards the base of the suckers that suggest expression within the cephalic ganglia (Fig. 4.5 C). Post-scolex, a line of *Hmic-Hh* expression runs centrally down through the entire midline of the worm from the very start of the neck and into mature segments (Fig. 4.5 A-B, D-E). Under higher magnification, this is shown to be a cluster of tightly packed, centralised cells, consistent with the genital primordia

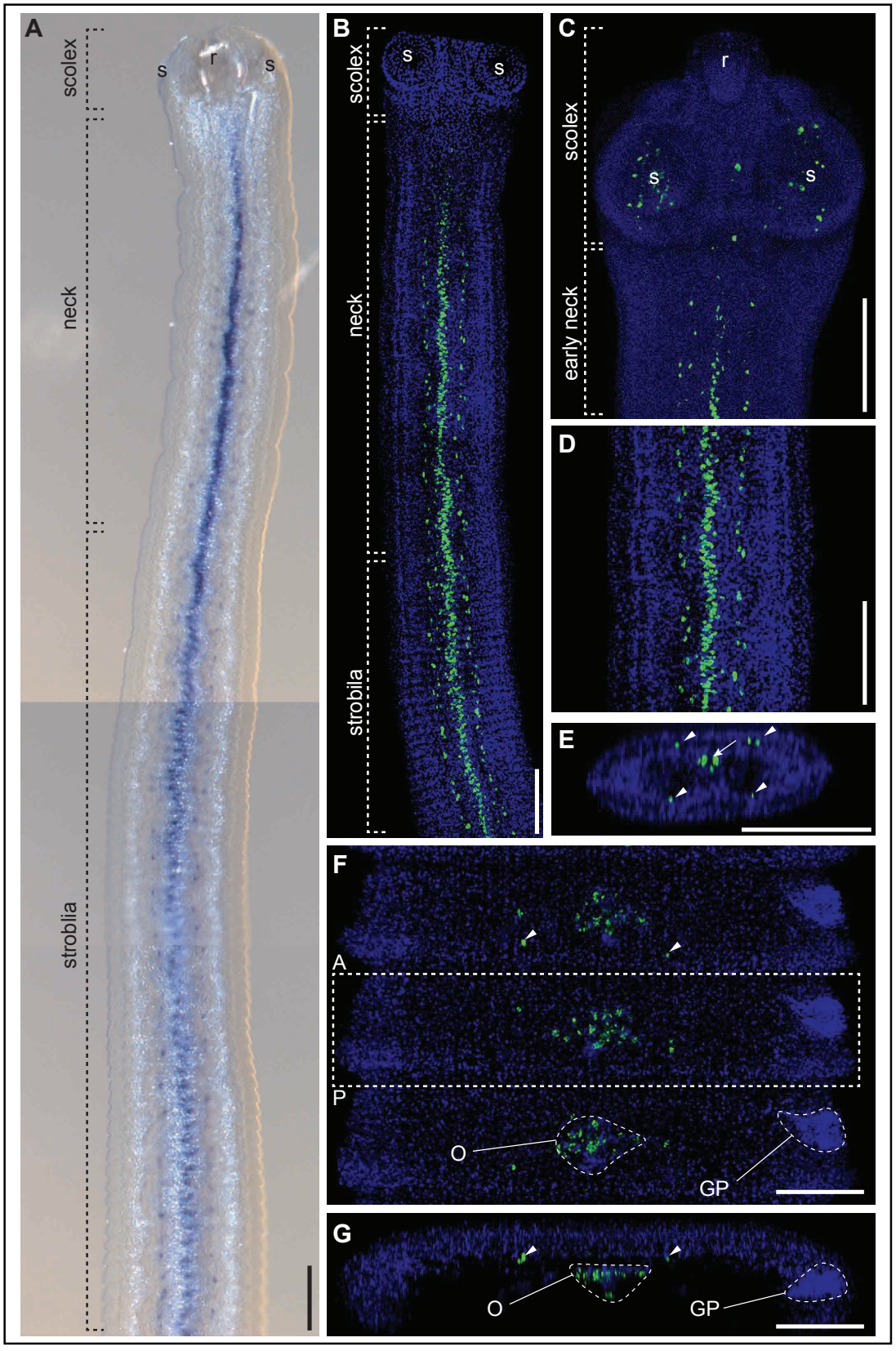

Figure 4.5 Expression of *Hmic-Hh* in adult *Hymenolepis microstoma*. A) Colorimetric ISH and B) maximum projection FISH in the adult worm showing a central stripe of expression surrounded by distinct foci that begins early in the neck and runs the length of the body. C) In the scolex, expression is

towards the base of the suckers and is activated early in the neck. Expression in D) the neck shows the central stripe to be the genital primordia and in E) cross section of the neck, this stripe is surrounded by four foci. F) In mature segments, *Hmic-Hh* expression is found in the ovary. Box designates one segment. G) In cross section, mature segments are shown to have one set of four foci of expression that mirror the location of the median nerve cords. Arrow heads indicate the four foci of expression close to the median nerve cords, arrow points to genital primordia. A = anterior, GP = genital pore, O = ovary, P = posterior, r = rostellum, s = suckers. Colorimetric whole-mount *in situ* hybridisation signal is shown in purple, FISH in green and DAPI in blue. Bars: 100 μm

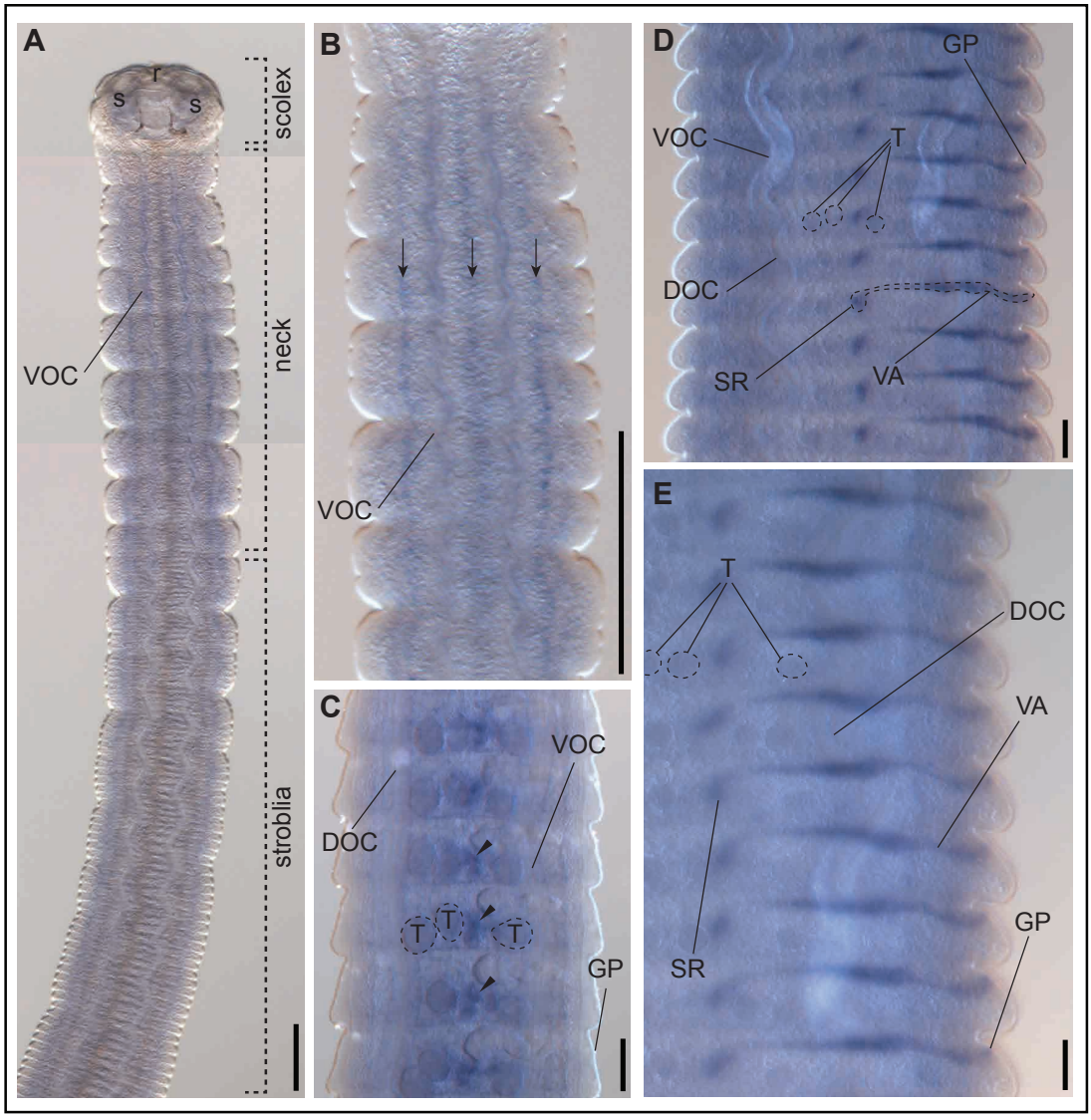

Figure 4.6 Expression of *Hmic-Ci* in adult *Hymenolepis microstoma*. Colorimetric ISH of *Hmic-Ci* shows weak expression across A) the adult worm. B) In the neck, stronger stripes of expression are observed (indicated by arrows). C) In mature segments, expression is strongest in the ovary (indicated by arrowheads). D) In immature segments expression is observed along the vagina and into the seminal receptacle. This can be seen more clearly in E) close up of immature segments. DOC = dorsal osmoregulatory canal, GP = genital pore, r = rostellum, s = suckers, SR = seminal receptacle, T = testes, VA = vagina, VOC = ventral osmoregulatory canal. Bars: 100 μm

(Wikgren, Gustafsson, and Knuts 1971; Koziol et al., 2010). As strobilation starts and proglottides begin to mature, this central stripe continues, eventually becoming condensed into clusters of cells within each segment. This central expression of *Hmic-Hh* is observed in both the developing and mature ovary (Fig. 4.5 F-G). As such, it is highly likely that the earlier expression of *Hmic-Hh* within the genital primordia (Fig. 4.5 E) is restricted to the female genital primordia. In the neck, the central stripe of *Hmic-Hh* expression is surrounded by four foci – two that are ventral-lateral, and two dorso-lateral that repeat along the length of the strobila (Fig. 4.5 A-B, E-G). The four foci are repeated at regular intervals and once strobilation begins, it becomes apparent that a set is located within each segment. The location of these four foci echoes the location of the junctions between the median and transverse nerve cords (Fig. 1.5) and it is possible that *Hmic-Hh* expression may be linked with the nervous system of *H. microstoma*.

Expression of *Hmic-Ci* is ubiquitous, although stronger patterns are also observed (Fig. 4.65. In the early neck, *Hmic-Ci* is expressed in several medial and lateral stripes. These stripes then cease towards the end of the neck (Fig. 4.6 A-B). The expression of *Hmic-Ci* is also linked with the female system. In segments, *Hmic-Ci* is observed along the vagina into the seminal receptacle and the ovary (Fig. 4.6 C-E).

Expression of *Hmic-Ptc* in adults is ubiquitous, although when observed using FISH, *Hmic-Ptc* appears punctate. In the neck, *Hmic-Ptc* is found in cortical cells and can be observed in a central stripe (Fig. 4.7 A). In mature

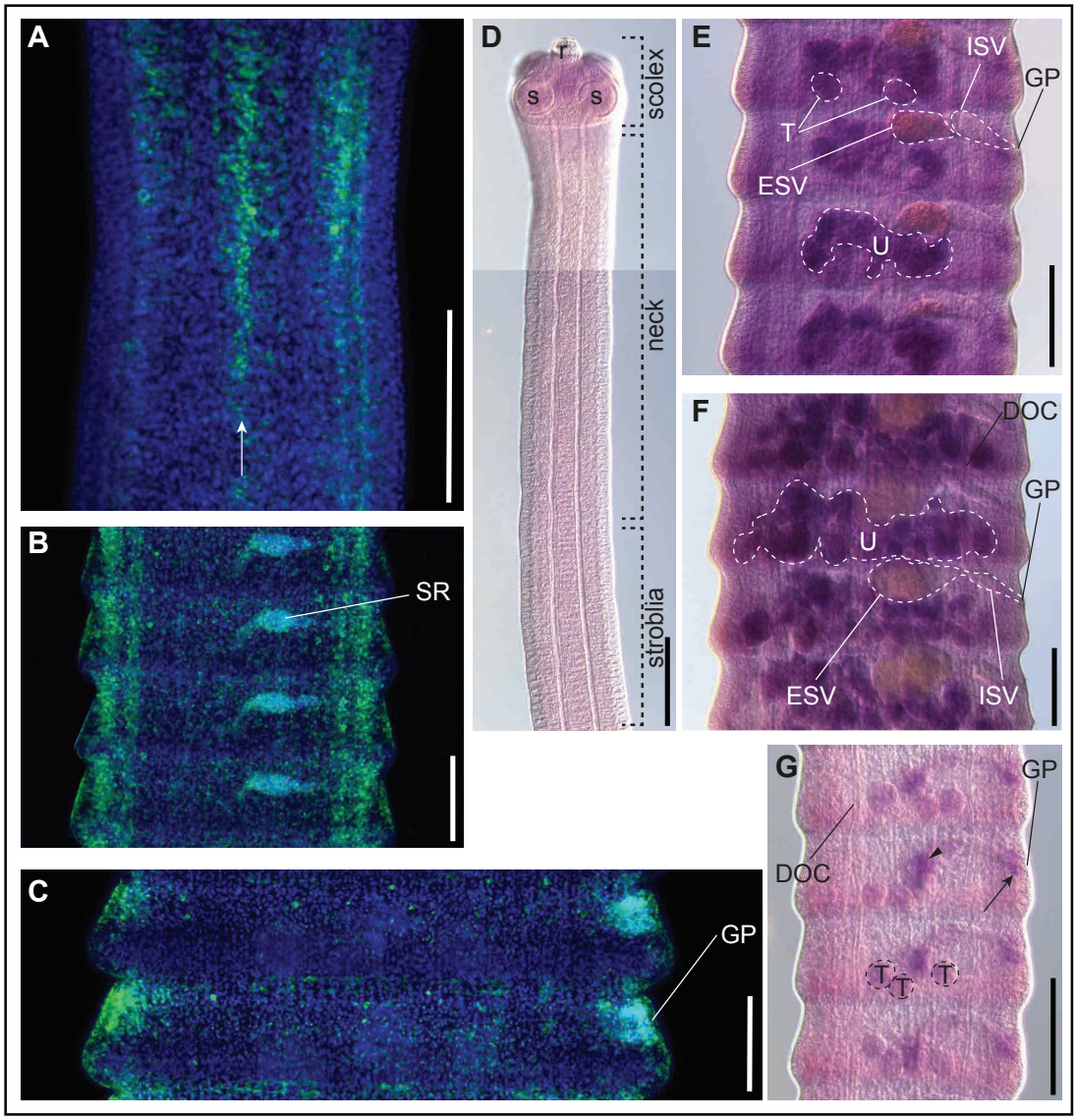

Figure 4.7 Expression of *Hmic-Ptc* and *Hmic-Sufu* in adult *Hymenolepis microstoma*. A) FISH of *Hmic-Ptc* in the neck, where the white arrow indicates a central stripe of expression. *Hmic-Ptc* in B) immature segments and C) mature segments. Colorimetric expression of *Hmic-Sufu* in D) the adult worm, E) mature segments, F) near-gravid segments and G) immature segments. Here darker staining can be seen surrounding the genital pore (indicated by black arrow) and in the ovary, indicated by the arrowhead. DOC = dorsal osmoregulatory canal, ESV = external seminal vesicle, ISV = internal seminal vesicle, GP = genital pore, r = rostellum, s = suckers, SR = seminal receptacle, T = testes, U = uterus. Colorimetric whole-mount *in situ* hybridisation signal is shown in purple, FISH in green and DAPI in blue. Bars: 100 μm

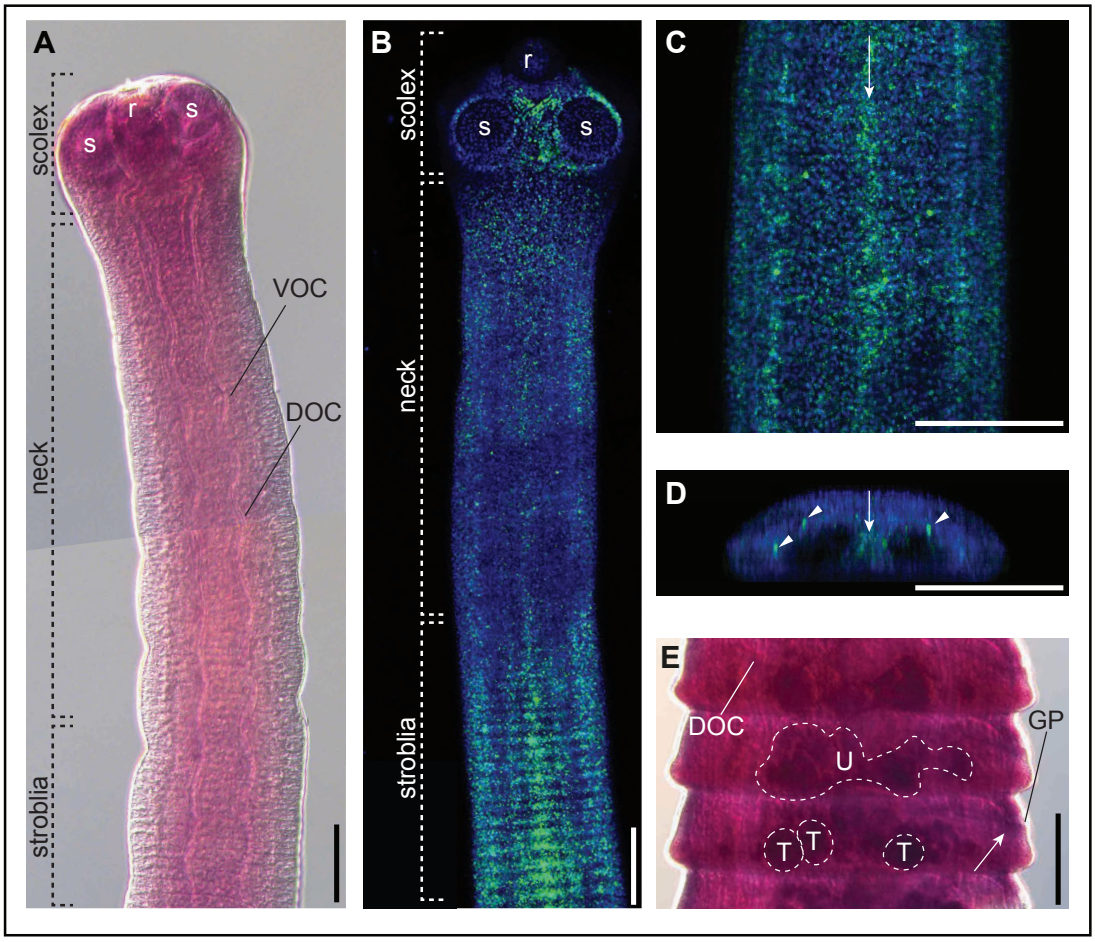

Figure 4.8 Expression of *Hmic-Smo* in adult *Hymenolepis microstoma*. Colorimetric ISH in A) the adult worm shows expression is ubiquitous. However, FISH in B) the adult worm, C) neck, D) neck in cross section indicates that this is punctate expression within the cortex. A central stripe of expression is observed in the neck (highlighted by arrows), as are medial-lateral foci (arrowheads). E) colorimetic ISH of mature segments shows stronger staining in the uterus. DOC = dorsal osmoregulatory canal, GP = genital pore, r = rostellum, s = suckers, T = testes, U = uterus, VOC = ventral osmoregulatory canal. Colorimetric whole-mount *in situ* hybridisation signal is shown in purple, FISH in green and DAPI in blue. Bars: 100 μm

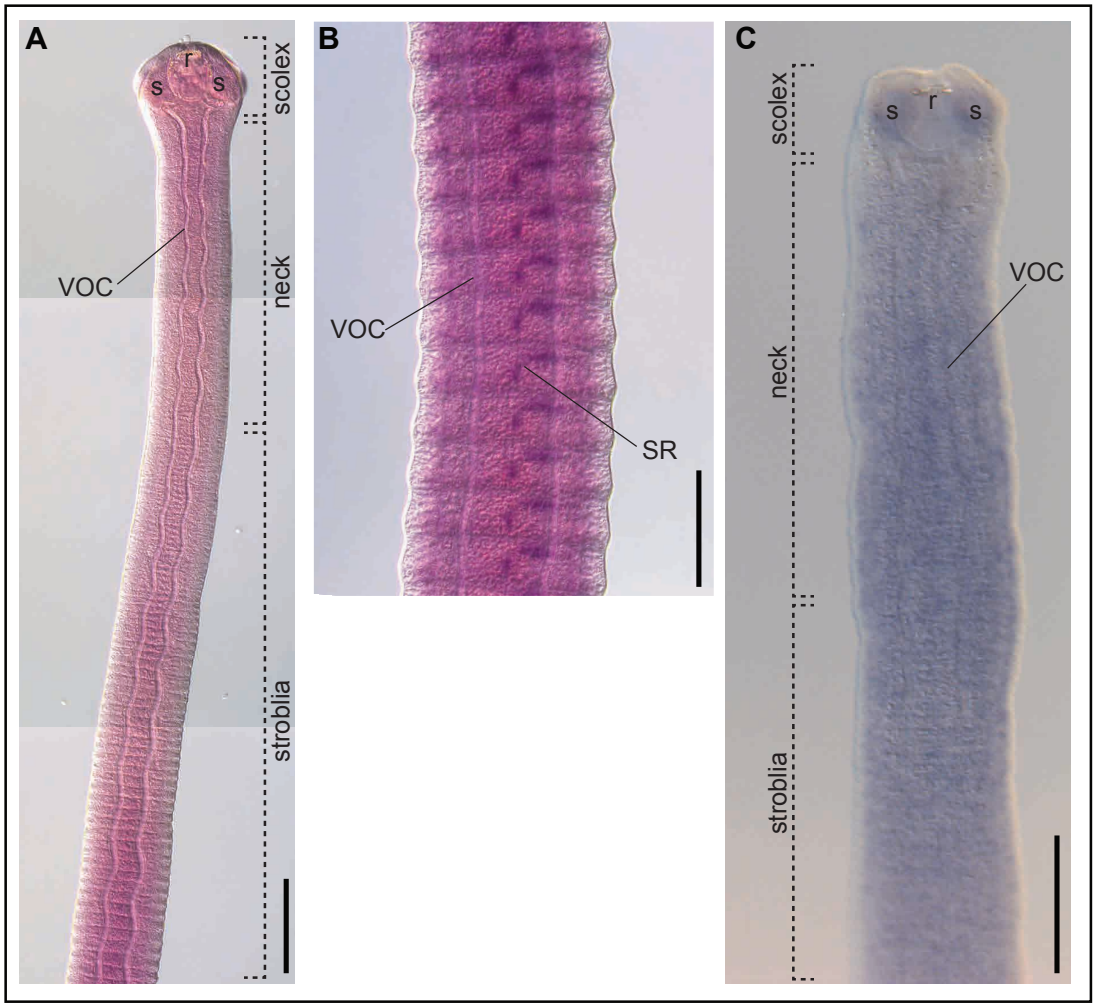

Figure 4.9 Expression of *Hmic-Slimb* and *Hmic-Disp* in adult *Hymenolepis microstoma*. Colorimetric ISH of *Hmic-Slimb* in A) the adult worm and B) immature segments shows weak, ubiquitous expression that is stronger in the seminal receptacle. C) Colorimetric ISH of *Hmic-Disp* in the adult worm is also ubiquitous. r = rostellum, s = suckers, SR = seminal receptacle, VOC = ventral osmoregulatory canal. Bars: 100 μm

segments, stronger expression is also observed in the seminal receptacle and surrounding the genital pore (Fig. 4.7 B-C).

Hmic-Smo expression is also ubiquitous and stronger in the uterus (Fig. 4.8), but there appears to be stronger expression centrally in the neck and in medial-lateral foci, as well as punctate expression within the cortex when observed using FISH.

Hmic-Sufu is weaker in the neck but is otherwise ubiquitous in adults (Fig. 4.7 D-G). Slightly stronger expression of *Hmic-Sufu* occurs in the ovary, seminal receptacle, genital pore and uterus. This again indicates a link between Hh signalling with the development of the female reproductive system and embryogenesis. The expression of *Hmic-Slimb* and *Hmic-Disp* are generally diffuse and ubiquitous (Fig. 4.9). In the neck, *Hmic-Slimb* expression appears to be weaker whilst in proglottides it is stronger in the developing seminal receptacle and ovary.

4.2.5 *Hmic-Hedgehog* expression is linked with the nervous system

When observed in cross section, four foci of *Hmic-Hh* expression are visible (Fig. 4.5 E) and these run the length of the strobila (Fig. 4.5 A-B, F-G). These four foci mirror where the median nerves run down the body of the worm (Fig. 1.5). An association between *Hmic-Hh* expression and the nervous system is also observed in the scolex (Fig. 4.5 C). Expression of *Hmic-Hh* in the scolex suggests localisation within the cephalic ganglia. These

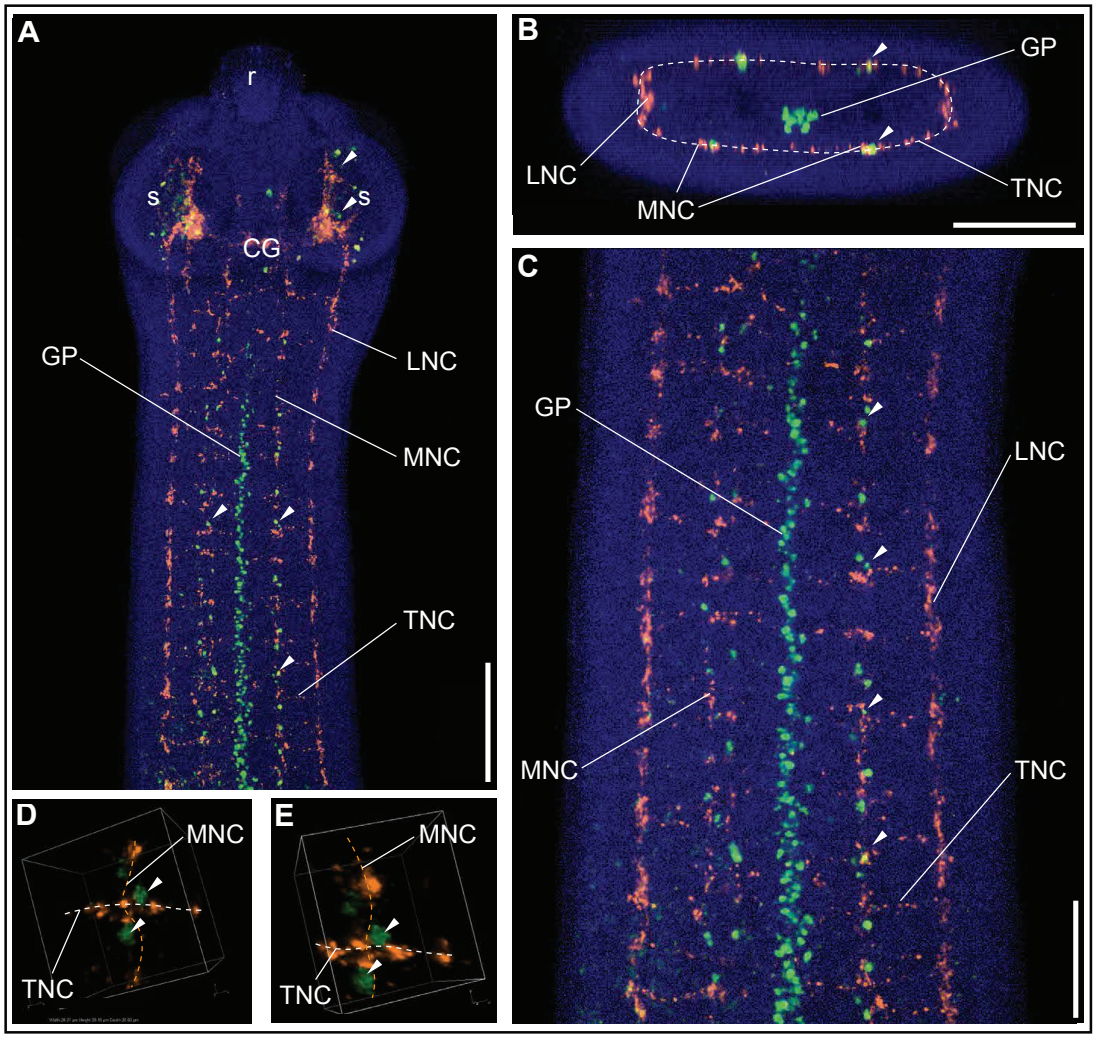

Figure 4.10 Expression of *Hmic-Hh* is associated with the nervous system in in adult *Hymenolepis microstoma*. Maximum projection of *Hmic-Hh* and immunostaining against synapsin in A) the scolex and early neck, B) the neck in cross section, C) the neck. Arrow heads indicate expression of *Hmic-Hh* closely associated with the median nerve cords. D) and E) High powered (x60) images of the commissures between transverse and median nerve cords in the neck. *Hmic-Hh* is expressed surrounding the junctions between transverse and median nerve cords. CG = cephalic ganglia, GP = genital primoria, LNC = lateral nerve cords, MNC = median nerve cords, r = rostellum, s = suckers, TNC = transverse nerve cords. Whole mount *in situ* hybridisation signal is shown in green, immunostaining against synapsin in orange and DAPI in blue. Bars: 100 μm

expression patterns indicate a link between this particular aspect of Hh signalling with the nervous system of *H. microstoma*. To confirm this, FISH of *Hmic-Hh* was conducted together with immunohistochemical staining against the broad neuronal marker, synapsin. The results from this analysis show that this is the case and that some adult expression of *Hmic-Hh* is associated with the nervous system (Fig. 4.10). In the scolex, expression co-localises in the lateral ganglia of the cephalic ganglia (Fig. 4.10 A). The medial-lateral nodes of *Hmic-Hh* expression (that are observed as four foci in cross section of the strobila) are associated with the median nerve cords. The foci are in line with the median nerve cords along the length of the strobila, and are almost always expressed near junctions with the main transverse nerves (Fig. 4.10 B-C). Under higher magnification (60x), this association is much clearer (Fig. 4.10 D-E). Along the median nerve cords, *Hmic-Hh* expression does not co-localise with staining against synapsin. Instead *Hmic-Hh* expression occurs adjacently to cells expressing synapsin, and is often observed at junctions between the median and transverse nerve cords, seemingly almost 'nestled' between the two nerve cords (Fig. 4.10 D-E).

4.3 Discussion

4.3.1 A conserved role for Hedgehog in neurogenesis

With the exception of *Hmic-Hh*, expression of Hh factors in adult *H. microstoma* are largely ubiquitous, with some stronger expression observed in the neck and female reproductive system. Median expression of *Hmic-Hh*

in the strobila is clearly closely linked with the tapeworm nervous system, as is expression within the cephalic ganglia (Fig. 4.10). *Hmic-Ci* expression in the neck may also be linked with the nervous system and/or the differentiation of stem cells prior to strobilation. Expression of *Hmic-Hh* is localised to the ventral nerve cords and medial border of the brain in the planarian, *Dugesia japonica*, whilst other Hh factors show ubiquitous expression (Yazawa et al., 2009). Expression of *Hh*, *Ptc*, *Smo*, *Sufu* and *Ci*/*Gli* in another planarian, *S. mediterranea*, mirrors these patterns observed in *H. microstoma* and *D. japonica* (Rink et al., 2009; Currie et al., 2016). *Hh* is observed in the CNS whilst *Ptc*, *Smo*, *Sufu* and *Ci* are ubiquitous throughout the worm. Some small variances are observed: slightly stronger expression is observed in the CNS (*Ptc* and *Smo*), pharynx (*Ptc*, *Smo* and *Sufu*) and gut epithelium (*Ci*) (Rink et al., 2009). The association of Hh signalling with the nervous system is clearly conserved within Platyhelminthes, as is the ubiquitous expression of other factors.

Double FISH showed *Hmic-Hh* to closely follow the line of the median nerve cords, however *Hmic-Hh* and synapsin staining did not co-localise (Fig. 4.10). Antibodies against synapsin act as a broad neuronal marker and stain the nervous system of tapeworms, however, it is not sufficient to observe the entire nervous system (Rozario and Newmark, 2015). Staining against synapsin in *H. microstoma* results in granular, punctate staining of the nerve cords under high magnification (Fig. 1.5) and other stains are required to resolve the finer structures of the nervous system (Koziol et al., 2013; Rozario and Newmark, 2015). Thus, *H. microstoma* cells expressing *Hmic-*

Hh may be part of the nervous system that staining against synapsin alone is unable to resolve. Wang et al. (2016) established that *S. mediterranea Hh* is expressed by neurons. Using the neuronal markers used by Wang et al. (2016) (*Pc2* and *Chat*) in *H. microstoma* may help to determine whether neurons are also expressing *Hh* in parasitic flatworms. Based on their close phylogenetic relationship, this is likely to be the case and we can assume that *H. microstoma* neurons express *Hmic-Hh*, just as their free-living relatives do. Recently, the presence of glia (neuronal support cells) were confirmed in planarians and RNAi experiments found that Hh signalling is required for the transcription of two genes (*If-1* and *Cali*) within these glial cells. This suggests that Hh signalling is required during the maintenance and regeneration of the planarian CNS (Wang et al., 2016). Tapeworms have a population of glia (Schmidt-Rhaesa et al., 2015) and given the similarities so far between Hh signalling in planarians and *H. microstoma*, the pathway may well be signalling to these CNS support cells in tapeworms, maintaining and coordinating neurogenesis both in proglottidees and the neck as the strobila extends.

Neurons producing *Hh* in the planarian brain communicate with dedicated neural neoblasts adjacent to the brain that express the receptor, Ptc. dFISH of the *S. mediterranea* neoblast marker *Piwi* (Reddien et al., 2005) with *Ci*/*Gli*, *Smo* and *Ptc* showed many stem cells next to the CNS to be the cellular targets of CNS-derived Hh signals (Currie et al., 2016). This may help explain the striped pattern of *Hmic-Ci* in the tapeworm neck, (some of which seem to follow the lines of nerve cords) (Fig. 4.5), an area rich with

germinative cells (Koziol et al., 2010). Constant signalling to these planarian stem cells is required to promote homeostatic neurogenesis of the CNS. Loss of Hh signalling leads to a reduction in the production of neural progenitors and neurons (Currie et al., 2016). In tapeworms, there is a close spatial association amongst nervous tissue, muscle and germinative cells (Wikgren et al., 1971; Halton and Maule, 2004; Koziol et al., 2010; Rozario and Newmark, 2015). It seems highly likely that a similar mechanism whereby neurons signal to closely positioned stem cells and neuronal support cells (i.e. glia) to control neurogenesis occurs in tapeworms as it does in planarians. The association between Hh signalling and the nervous system is conserved more broadly amongst flatworms with vertebrates and *D*. *melanogaster*. *Hh* is expressed in the neural floor plate of vertebrates (Dessaud et al., 2008) whilst in *D. melanogaster*, Hh signalling regulates glia cell fate, inducing different subtypes of glia (Watson et al., 2011).

4.3.2 Hedgehog signalling and development of the female reproductive system

A second pattern of *Hmic-Hh* expression is observed throughout larval development and in adult *H*. *microstoma* that is not associated with the nervous system. This pattern is more in line with another conserved feature of Hh signalling - patterning of the midline. This pattern is observed in molluscs (Nederbragt et al., 2002) and vertebrates (Krauss et al., 1993; Martí et al., 1995; Roelink et al., 1995). However, this expression is linked with the development of the neural floor plate and notochord (Dessaud et al., 2008).

Whilst part of *H. microstoma* Hh signalling (in adults) is clearly linked with the nervous system, midline patterning of the tapeworm is not. Firstly, whilst the expression observed in the fully developed cysticercoid may be within the cephalic ganglia of the newly formed scolex, the developing oncosphere lacks an integrative nervous system, with no brain. Even the presence of nerve cells is controversial. Recently, two putative nerve cells have been described in oncospheres (Fairweather and Threadgold, 1981; Hartenstein and Jones, 2003; Jabbar et al., 2010; Młocicki et al., 2010). Second, in the adult worm, nerve cords do not run through the centre of the worm (Fig. 1.5). Instead this central stripe of expression in the neck is likely to represent the genital primordia and stem cells involved in the process of proglottisation. Later expression of *Hmic-Ci* and *Hmic-Hh* occurs in the developing seminal receptacle and ovary, indicating that Hh signalling and activation of downstream targets is required for development of the female reproductive system in tapeworms. Hh signalling is vital for ovarian development and oogenesis by regulating cell proliferation and differentiation of ovarian stem cells in *D. melanogaster* (Forbes et al., 1996; Tworoger et al., 2017; Zhang and Kalderon 2000; Zhang and Kalderon 2001; Kirilly and Xie, 2007) and mice (Wijgerde et al., 2005; Russell et al., 2007). Whilst in ovarian cancers, aberrant over activation of Hh signalling, resulting in the up-regulation of *Hh*, *Ci*, *Ptc* and *Smo*, is observed (Chen et al., 2007; Bhattacharya et al., 2008; Liao et al., 2008). Most research relating to Hh signalling in ovarian development is directed towards its role in cancer, however, it is interesting that this feature may be conserved across flatworms, arthropods and vertebrates.

4.3.3 Hedgehog and larval development

Whilst the role of Hh signalling in midline development can be explained in cysticercoids and adult worms, the situation is less clear during larval development. Whilst Hh signalling is likely to be involved in the patterning of the larval midline, exactly what cell type *Hmic-Hh* is expressed by is unclear. Larval tapeworm nerves are presumed to develop laterally and do not form until later stages of development. Therefore, given the expression of *Hmic-Hh* along the midline of *H. microstoma* larvae, it is highly unlikely that *Hmic-Hh* is associated with nervous tissue, or even neural precursors. Oncospheres also lack a brain and so anterior expression cannot be linked with this structure.

The expression of other Hh factors in larvae prevents a meaningful interpretation of the potential role of Hh signalling during larval development. The posteriorised expression of many of these factors during mid development and later observation within the cyst tissue could indicate a potential role in guiding AP axis formation. However, Hh signalling could also be playing many roles simultaneously. As such, further analysis is still required.

4.3.4 General conclusions and future directions

Overall, the presence and expression of Hh factors in *H. microstoma* shows canonical Hh signalling to be present and conserved within tapeworms.

Expression in adults suggests that the pathway is involved in two processes simultaneously – neurogenesis and proglottisation. Based on expression patterns, it is unlikely that Hh signalling is involved in strobilation, as *Hmic-Hh* turns on before this process begins and no polarised expression of *Hmic-Hh* (or other pathway factors) is observed in individual segments. Therefore, *Hmic-Hh* does not act as a segment polarity gene as it does in *D. melanogaster* (or other arthropods). The repeated expression of *Hmic-Hh* in every segment is more likely due to the segmented nature of the tapeworm nervous system. Hh signalling is active during proglottisation (that begins almost instantly in the neck) and is likely to be guiding both proglottisation and organogenesis. Future work is required to further understand Hh signalling in larvae and the use of now-known neuronal markers in planarians will aid in confirming that tapeworm neurons express *Hmic-Hh*. Ultimately the development of robust functional tools is required to confirm the role of Hh signalling in *H. microstoma*.

Chapter 5

The Wnt pathway in *Hymenolepis microstoma*

Elements of this chapter were published as part of: ‘Comparative analysis of Wnt expression identifies a highly conserved developmental transition in flatworms’, BMC Biology, 2016. U. Koziol, F. Jarero, P.D. Olson & K. Brehm (Appendix 5)

5.1 Introduction

The Wnt pathway (Fig. 5.1) is a highly conserved cell-cell signalling system that controls many developmental processes, including cell-fate determination, polarity, patterning, morphogenesis, cell proliferation, migration and apoptosis (Cadigan and Nusse, 1997; Martin and Kimelman, 2009; Petersen and Reddien, 2009). Wnt signalling controls anteroposterior (AP) axis patterning in many metazoans (Petersen and Reddien, 2009) and the segmentation of many arthropods, annelids and vertebrate somitogenesis. As such, the pathway is likely to control AP patterning in tapeworms and is a strong candidate with which to investigate strobilation.

Historically, Wnt ligands have been categorised to transduce three discrete pathways depending on whether or not they lead to the activation of *β-catenin*. These are the canonical β-catenin dependant pathway and the non-canonical planar cell polarity (PCP) and Wnt/calcium pathways. However, the classification of these different pathways (or cascades) may be artificial, with Wnt signalling proving to be a highly complex and dynamic system in which cross-talk between the different cascades occurs (van Amerongen and Nusse, 2009). These authors suggest that Wnt signalling should not be thought of as a linear pathway and instead should focus on context-specific interactions between Wnts and their receptors. Despite this, the best understood transduction cascade remains the canonical pathway, which has a conserved role involved in the specification of the anteroposterior (AP) and primary axes. This chapter focusses on the role of β-catenin dependant

signalling in the development of *H. microstoma*. The genes of the pathway have already been characterised (Riddiford and Olson, 2011) and this chapter will centre on the expression of some of these factors through *in situ* hybridisation experiments during larval and adult development.

5.1.1 Wnt discovery

The first mammalian *Wnt* gene (*Int1*) was discovered in the early 1980's during screens identifying tumour growth in mice (Nusse and Varmus, 1982). The impact of *Int1*'s discovery was overshadowed by a rapid influx of many other developments in cancer biology at the time (Nusse and Varmus, 2012). Despite this, the sequence and structure of *Int1* was characterized (van Ooyen and Nusse, 1984), as was its cDNA sequence (Fung et al., 1985) which showed no homology to any other gene at the time (Nusse and Varmus, 2012). Around the same time, screening in *Drosophila melanogaster* identified several 'segment polarity genes' (so-called because mutant flies presented abnormal segmental patterning), one of which was the gene *Wingless* (*Wg*). *Wg* mutants were found to lack segment boundaries (Nusslein-Volhard and Wieschaus, 1980). Previously identified in earlier developmental studies, *Wg* mutations resulted in wingless or haltere-less flies and other developmental deformities in the mesothorax (Sharma and Chopra, 1976). Further investigations uncovered *Int1* and *Wg* to in fact be orthologous genes (Rijsewijk et al., 1987). As interest in the gene took hold, other groups began to indicate a role in embryonic axis formation (McMahon and Moon, 1989). Early knockouts of *Int1* in mice caused anteriorised

defects during development, such as a reduced cerebellum (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). During the early 1990's the nomenclature of *Int* and *Wg* was becoming confusing and the names were combined to become '*Wnt*' (Nusse et al., 1991). Since then, the Wnt pathway has been identified as a highly conserved signalling pathway, found throughout the Metazoa (Kusserow et al., 2005) with a clear ancestral role in forming the primary axis of animals (Petersen and Reddien, 2009; Niehrs, 2010; Holstein et al., 2011; Holstein, 2012).

5.1.2 Mechanism of action

For a general overview of the mechanism of action of the Wnt pathway, see Fig. 5.1. *Wnts* encode secreted glycoproteins that bind to members of the Frizzled (Fz) receptor family, proteins with seven transmembrane regions and similarities with G-coupled receptors (Schulte and Bryja, 2007). Before Wnts are released and can bind to Fzs, they must undergo modification, including glycosylation and palmitoylation (Smolich et al., 1993; Willert et al., 2003; Takada et al., 2005). Palmitoylation is mediated by Porcupine and is essential for the maturation of Wnts (Kadowaki et al., 1996; Hofmann, 2000). The loss of *Porcupine* can lead to defects in Wnt secretion in *D. melanogaster* (reviewed in Cadigan and Nusse 1997). Lipid modification of Wnts are thought to aid in efficient signalling and the secretion of the ligands (Willert et al., 2003; Kurayoshi et al., 2007; Franch-Marro et al., 2008). Secretion of Wnts are further regulated by Wntless, whose action is still unclear, but binds Wnt in the Golgi apparatus (Bänziger et al., 2006) and is

[Figure removed due to copyright]

Figure 5.1 The Wnt signalling pathway. A) Wnt modification and secretion. B) Wnt signalling – binding with the receptor. C) Wnt signalling in the nucleus – transcription of target genes. See image text and chapter for a more detailed description of the pathway's mechanism of action and factor interactions. (Adapted from Clevers and Nusse, 2012)

thought to act as a 'sorting receptor' (Port et al., 2008; Clevers and Nusse, 2012). Once secreted, Wnts can bind to Fzs. An individual Wnt ligand from any sub-family can bind to multiple Fz receptors, and vice versa (Bhanot et al., 1996). The Wnt-Fz interaction along with the co-receptor Low density lipoprotein receptor related 5/6 (LRP5/6) confers a conformational change that allows Axin to bind to the cytoplasmic tail of LRP5/6 (Mao et al., 2001; Zeng et al., 2008). The binding of LRP5/6 to axin is catalysed by phosphorylation of LRP5/6 by the kinases Glycogen synthase kinase 3 (GSK3) and Casein kinase 1 (CK1). After binding of Wnt to the receptor complex, Dishevelled (Dsh) is recruited and it is at this point that Wnt signalling diverts into one of the three discrete pathways (Komiya and Rabas, 08). In canonical signalling, binding of Wnt to the receptor complex induces the association of the APC/Axin/GSK3 complex (the destruction complex) with LRP5/6. The destruction complex is required for the degradation of β-catenin (Gordon and Nusse, 2006). The interaction of the destruction complex with Dsh prevents β-catenin degradation instead, it stabilises and accumulates in the cytoplasm (Hatsell et al., 2003). β-catenin then translocates to the nucleus where it binds to TCF/LEF, mediating the transcription of target genes. In the absence of Wnt, the APC/Axin/GSK3 destruction complex is able to bind and phosphorylate β-catenin which is then degraded, preventing its accumulation and subsequent up-regulation of target genes. In the nucleus, TCF/LEF helps to repress gene transcription by binding with Groucho instead of β-catenin.

Secreted antagonists can inhibit Wnt signalling in numerous ways (reviewed in MacDonald et al., 2009). Firstly, by soluble/secreted frizzled-related proteins (sFRPs), Wnt inhibitory factor (Wif) and Cerberus that bind to Wnt in the extra cellular matrix, preventing interactions with Fz (Hoang et al., 1996; Hsieh et al., 1999; Bovolenta et al., 2008). sFRPs are also found to be able to bind to Fz receptors as well as the Wnt ligands themselves (Bafico et al., 1999; Carron et al., 2003; Rodriguez et al., 2005). This suggests that sFRPs can also inhibit Wnt signalling in ways other than acting as a classical antagonist (Bovolenta et al., 2008). Secondly, by the Dickkopf family (Glinka et al., 1998) and members of the WISE/SOST family that bind to the co-receptor LRP5/6, preventing completion of the Wnt-Fz-LRP5/6 complex. It is thought that these two classes of factors help create a gradient of Wnt activity, limiting its range (Kawano and Kypta, 2003).

5.1.3 Role of Wnt signalling in regulating anteroposterior polarity

A striking role of Wnt signalling is its involvement in establishing the primary body axes in many metazoans. *Wnt* repression results in anteriorisation and this function is highly conserved and likely to predate the split between the cnidarians and bilaterians (Petersen and Reddien, 2009; Niehrs, 2010). No *Wnts* or their receptors are present in choanoflagellates (King et al., 2008). In many species, *Wnts* are expressed towards and are involved in specifying the posterior (Petersen and Reddien, 2009). Expression of *Wnt* in the sponge, *Amphimedon queenslandica*, is observed in the posterior of larvae (Adamska et al., 2010) whilst *Wnts* in the cnidarian *Nematostella vectensis*,

are expressed in overlapping patterns along the oral-aboral axis from the middle to the oral pole (Kusserow et al., 2005). Recent findings have also identified *β-catenin* is involved in refining patterning of the oral-aboral axis in *N. vectensis* (Lèclere et al., 2016). However, *β-catenin* does not function along a gradient of expression as it does in bilaterians and is not likely to be required for initial establishment of oral-aboral polarity. The exact mechanism of Wnt/β-catenin signalling in *N. vectensis* is yet to be determined (Lèclere et al., 2016).

Wnt signalling has not been shown to be involved in AP patterning of *Drosophila*. However, evidence suggests that it does in other arthropods. Three *Wnts* (*Wnt1*, *Wnt8/D* and *WntA*) are expressed at the posterior of the blastoderm stages of *Tribolium castaneum* during embryogenesis suggesting a role in posterior patterning (Bolognesi et al., 2008a). Subsequent knockdown of *Wnt8/D* (via RNAi) resulted in a loss of abdominal segments in some embryos. The effects of which were stronger when combined with *Wnt1* RNAi (Bolognesi et al., 2008b). These studies confirm that Wnt signalling is also involved in patterning of the posterior in non-dipteran arthropods.

Wnt/β-catenin signalling is also required for axial patterning in vertebrates. Post-gastrulation, canonical Wnt signalling is directed towards specification of the AP axis. As in the specification of the AP axes of other metazoans (see Petersen and Reddien, 2009), several vertebrate *Wnts* (including *Wnt3a*, *Wnt5a*, *Wnt8* and *Wnt11*) are considered ‘posterior’ and are

expressed in the posterior of the developing embryo (Krauss et al., 1992; Moon et al., 1993; Kelly et al., 1995; Hong et al., 2008; Schier and Talbot, 2005; Hikasa and Sokol, 2013). Conversely, Wnt antagonists (such as *Sfrp3*) are expressed anteriorly (Leyns et al., 1997; Wang et al., 1997; Houart et al., 2002; Schier and Talbot, 2005). Functional studies in vertebrates have confirmed that these expression patterns are linked with a posterior role and its inhibition leading to anteriorisation (Petersen and Reddien, 2009; Hikasa and Sokol, 2013. *Wnt/β-catenin* gradient is responsible for regulating early patterning of the *Xenopus* central nervous system (CNS), with increased levels of *β-catenin* found anteriorly (Kiecker and Niehrs, 2001). Meanwhile, antagonism of Wnt signalling inhibits its posteriorising effect, resulting in the formation of ectopic eyes and over-growth of the fore-brain (Richard-Parpaillon et al., 2002). In zebrafish, loss of *Wnt8* leads to defect in the AP polarity of neural tissue (Erter et al., 2001; Lekven et al, 2001). Simultaneous inhibition of *Wnt8* and *Wnt3a* exacerbates the loss of posterior identity with the loss of posterior body structure and the anteriorisation of neuroectoderm (Shimizu et al., 2005). Canonical Wnt signalling is also required for development of posterior structures in mice (reviewed in Grigoryan et al., 2008).

5.1.4 Wnt and segmentation

Wnt was originally described as a ‘segment polarity gene’ (Nusslein-Volhard and Wieschaus, 1980) and works to direct the polarity of *D. melanogaster* segments and vertebrate somites (Baker, 1987; Diaz-Benjumea and Cohen,

1995; Aulehla et al., 2003; Pourquié, 2003; Aulehla and Herrmann, 2004; Dequéant et al., 2006). In *Drosophila*, *Wg* acts towards the end of the segmentation process, helping to establish segment polarity by defining parasegment boundaries. After a hierarchical expression cascade of maternal effect, gap and then pair-rule genes, *Wg* expression is activated and observed in a striped manner in the *Drosophila* embryo. Cells expressing *Wg* maintain the expression of another 'segment polarity gene' – *Engrailed* (*En*) – in posteriorly bordering cells. *En* expressing cells transcribe and posteriorly secrete *Hh* which, in turn, maintains *Wg* expression. The boundary between *Wg* and *En* defines the parasegment boundary with anterior *En* and posterior *Wg* expression within each parasegment. Segment boundaries are then formed posteriorly to the *En* expressing cells (reviewed in Seaver, 2003; Swarup and Verheyen, 2012).

The role of the 'segment polarity genes' shows the strongest conservation amongst arthropods, and is thought to represent the 'phylotypic stage' of development (2002; Seaver, 2003; Peel et al., 2005). The role of Wnt signalling to form segment boundaries is conserved in non-dipteran arthropods. In non-dipteran insects, *Wnt* expression is broadly similar to that of *Drosophila* (Seaver, 2003), with *Wnt*s observed in striped patterns along segment borders and knockdown resulting in segmentation defects (Angelini and Kaufman, 2005; Bolognesi et al., 2008b; Janssen et al., 2010; Chesebro et al., 2012). In crustaceans, Wnt signalling is still involved in the segmentation process, however it seems to rely on multiple *Wnt* orthologs (reviewed in Seaver, 2003).

In some lophotrochozoans, *Wnts* are expressed in segmentally repeated patterns (Cho et al., 2010; Janssen et al., 2010). However, in another annelid, *Capitella* sp. I, *Wnt1* is only expressed after morphological segmentation, indicating it is not involved in segmentation (Seaver and Kaneshige, 2006).

5.1.5 Planarian Wnt signalling

Similar to the role observed during early development of other bilaterians, canonical Wnt signalling has a clear involvement in both the establishment and maintenance of the AP axis of planarians (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008; Petersen and Reddien, 2009a and b; Almuedo-Castillo et al., 2012). *Wnts* are expressed in gradients from the posterior of the animal and are required for specification of the planarian posterior. Antagonists of the system are expressed towards the anterior and Wnt inhibition is required for anterior specification.

A long-standing question in planarian biology was how an injured worm knows whether to develop a head or tail. The first evidence linking canonical Wnt signalling with a role in planarian AP development came about through RNAi experiments that knocked down *β-catenin-1*. Silencing *β-catenin-1* in *S. mediterranea* results in an anteriorised worm with the loss of posterior (and central) identities (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). These individuals are radially symmetrical with hypercephalisation in the most extreme cases, whilst decreasing the *β-*

catenin-1 dsRNA concentration results in weaker phenotypes. These range from two-headed planarians with ectopic eyes to so-called 'tailless' planarians (Iglesias et al., 2008; Adell et al., 2010). It was the discovery of the role *β-catenin* plays during planarian regeneration that provided the catalyst that has sparked the intensive research of planarian biology taking place today.

Planarians have nine *Wnts* from four different subclasses –individual copies of *Wnt1*, *Wnt2* and *Wnt5* with the remaining *Wnts* belong to the *Wnt11* subclass (Petersen and Reddien, 2008; Adell et al., 2009; Gurley et al., 2010). *Wnt1* is calssified as a 'posterior' *Wnt* and is expressed posteriorly along the dorsoventral midline (Petersen and Reddien 2008; Gurley et al. 2010). Silencing of *Wnt1* results in 'tailless' animals and in a small minority, 'two-headed' planarians (Adell et al., 2009; Almuedo-Castillo et al., 2011). Several *Wnts* belonging to the *Wnt11* subclass are also classified as 'posterior' *Wnts* along with *Wnt1* – *Wnt11-1*, *Wnt11-2* and *Wnt11-5* (Sureda-Gómez et al., 2015). These are all expressed posteriorly (Petersen and Reddien, 2008, 2009; Adell et al., 2009) and simultaneous silencing (rather than individual) generates the strongest anteriorised phenotypes (Sureda-Gómez et al., 2015). *Wnt2* is observed laterally in the head, whilst *Wnt5* is expressed laterally along the dorsoventral boundary (Adell et al., 2009; Gurley et al., 2010; Almuedo-Castillo et al., 2011).

Developmental control genes have been proposed as factors that act as a Cartesian coordinate system (Niehrs, 2010) during the maintenance of tissue

in planarians (Reddien, 2011). These genes are regionally expressed along body axes and they either involved in the regulation of signalling pathways or produce aberrant phenotypes when inhibited. Muscle cells have recently been confirmed as the source of these developmental control genes, that they termed 'positional control genes' (PCGs) (that include *Wnts*, *Sfrps* and *Fz4*), in planarians (Witchley et al., 2013). It is these muscle cells that provide positional information to pluripotent neoblasts during planarian regeneration and tissue turnover by acting as 'landmarks' during the differentiation of new and missing tissue.

5.1.6 *Wnt* gene loss in flatworms

A bioinformatic study into the presence of *Wnts* across the Platyhelminthes (Riddiford and Olson, 2011) found that the major signalling factors (from all three discrete pathways) are present in *H. microstoma* (and other flatworms) except *Dickkopf* and *Cerberus* (Table 5.1). The presence of the full complement of Wnt components suggest that Wnt signalling is functional in *H. microstoma*. There are some notable gene losses, however, within the phylum. The complement of *Wnts* themselves is highly reduced in all flatworms (Riddiford and Olson, 2011; Tsai et al., 2013) with orthologs present from only five of thirteen sub-families (Riddiford and Olson, 2011). There are fewer paralogs generally in parasitic species compared with free-living ones: six in cestodes, five in the blood fluke, *Schistosoma mansoni*, and nine in the planarian, *Schmidtea mediterranea*.

5.2 Results

5.2.1 Further identification of Wnt factors

Clear orthologs of *Wnts* and other genes related to the pathway are present in *H. microstoma* (Riddiford and Olson, 2011) (Table 5.1). Further analysis identified a sFRP-like gene with a divergent netrin domain, dubbed *Hmic-Sfl* (Koziol et al., 2016). A second paralog of *GSK3* that is also involved in Hh signalling (Table 4.1) and *Dsh* were also found (Table 5.1).

5.2.2 Larval expression

5.2.2.1 *Wnts* are expressed towards the posterior of developing larvae

During early larval development, *Hmic-Wnt11a* is expressed diffusely in the hook-bearing half of the worm, in lateral stripes in the central region and in two cells at the hook-bearing pole (Fig. 5.2 D). The expression in these two cells remains turned on throughout development into the metacestode. As the larva continues to develop, expression becomes less diffuse and the lateral stripes running from the centre to posterior of the worm become more apparent. Once the larvae have encysted, the lateral stripes have disappeared and nodes of expression are observed at the apex of the cyst tissue opposing the hooks (Fig. 5.2 D). *Hmic-Wnt11b* is also initially expressed diffusely in the posterior half of stage I larvae and in two cells in the hook-bearing pole of the worm (Fig. 5.2 E). Expression is strongest in

Table 5.1 Wnt pathway factors in *Hymenolepis microstoma*. Wnt pathway factors, their predicted gene model numbers and the length of predicted proteins in base pairs. RNA-Seq data for each gene model is given in the number of reads per kilobase per million mapped reads (RPKM) for four stages of development – mid larval stages, whole adult, the scolex/neck, mid (i.e. mature segments) and end (i.e. gravid segments). Differential expression between regions is given (from Riddiford and Olson, 2011).

Gene	Gene model	Length (bp)	RPKM values					Differential expression			
			Larva	Whole adult	Scolex/ neck	Mid regions	End regions	Larva vs whole adult	Scolex/neck vs mid	Scolex/neck vs end	Mid vs end
Hmic-Wnt1	HmN_000328000	1569	0.4	20.6	22.6	6.1	7.1	UP	ND	DOWN	ND
Hmic-Wnt2	HmN_000112200	1590	3.7	2.7	3.5	1.6	1.8	ND	ND	ND	ND
Hmic-Wnt4	HmN_000808600	1497	164.6	21.8	14.5	10.7	12.2	DOWN	ND	ND	ND
Hmic-Wnt5	HmN_000108100	1119	18.3	23.9	16.1	5.9	9.3	ND	ND	ND	ND
Hmic-Wnt11a	Not predicted										
Hmic-Wnt11b	HmN_000022800	1401	29.6	38.9	48.4	21.4	16.5	ND	ND	DOWN	ND
Hmic-FrizzledA	HmN_000386300	2247	19.6	42.1	51.7	22.3	21.7	ND	ND	DOWN	ND
Hmic-FrizzledB	HmN_000319700	2241	180.6	48.2	112.5	28.8	20.7	ND	DOWN	DOWN	ND
Hmic-FrizzledC	HmN_000494400	1743	0.7	11.7	9.9	4.8	3.2	UP	ND	DOWN	ND
Hmic-FrizzledD	HmN_000227100	1974	217.7	38.1	71.6	25.3	21.4	DOWN	DOWN	DOWN	ND
Hmic-FrizzledE	HmN_000595200	1566	245.6	25.3	13.8	15.0	17.0	DOWN	ND	ND	ND
Hmic-Dishevelled-A	HmN_000446600	2478	45.7	25.2	12.6	15.6	13.1	ND	ND	ND	ND
Hmic-Dishevelled-B	HmN_000723000	2955	39.7	40.3	12.9	24.8	17.7	ND	ND	ND	ND
Hmic-APC	HmN_000462000	6552	95.9	36.9	16.6	13.7	15.0	ND	ND	ND	ND
Hmic-Axin	HmN_000572400	2754	45.2	21.5	5.6	17.8	7.3	ND	UP	ND	ND
Hmic-βCatenin A	HmN_000161700	3195	304.1	99.4	71.9	60.3	42.8	ND	ND	DOWN	ND
Hmic-βCatenin B	HmN_000192700	1956	21.1	6.8	14.9	7.5	3.9	ND	ND	DOWN	ND
Hmic-LEF1/TCF	HmN_000448500	3627	26.8	8.2	3.1	4.0	2.7	ND	ND	ND	ND
Hmic-Wif	HmN_000932100	777	18.7	20.0	26.3	19.3	7.3	ND	ND	DOWN	ND
Hmic-Sfrp	HmN_000556500	1704	29.7	64.5	17.6	16.7	24.5	ND	ND	ND	ND
Hmic-Sfl	HmN_000359400	1746	137.0	130.3	138.4	72.4	88.5	ND	ND	DOWN	ND

these two cells and stays on during later stages of development. Once encysted, *Hmic-Wnt11b* remains distinctly within the cyst tissue (Fig. 5.2 E). The expression of *Hmic-Wnt1* is generally ubiquitous and diffuse at first (Fig. 5.2 C). As metamorphosis continues the expression of *Hmic-Wnt1* becomes gradually restricted to the posterior and the cyst tissue as the worm develops into the metacestode (Fig. 5.2 C).

5.2.2.2 Antagonists are expressed anteriorly in developing larvae

The Wnt inhibitor *Hmic-Sfrp* is expressed in a small cluster of cells at the pole that develops into the scolex (Fig. 5.2 A). This continues throughout larval development; as the larva extends, this small group of cells at the anterior apex continues to express *Hmic-Sfrp*. Once the anterior of worm has been withdrawn into the cyst, the only cells expressing *Hmic-Sfrp* continue to be the small apical cluster situated within the developing rostellum during stages IV and V and remains turned on in the metacestode (Fig. 5.2 A). A second antagonist, *Hmic-Sfl*, is also found in a tight cluster of cells in the pole that goes on to develop into the scolex (Fig. 5.2 B). Their expression differs in that the *Hmic-Sfl* cluster is larger than that of *Hmic-Sfrp* and also extends laterally in two stripes that stop short of the hook-bearing pole. The lateral stripes and a small apical cluster remain during the mid-stages of larval development and once encysted, expression is observed in many cells throughout the tissue of the encysted juvenile worm (Fig. 5.2 B).

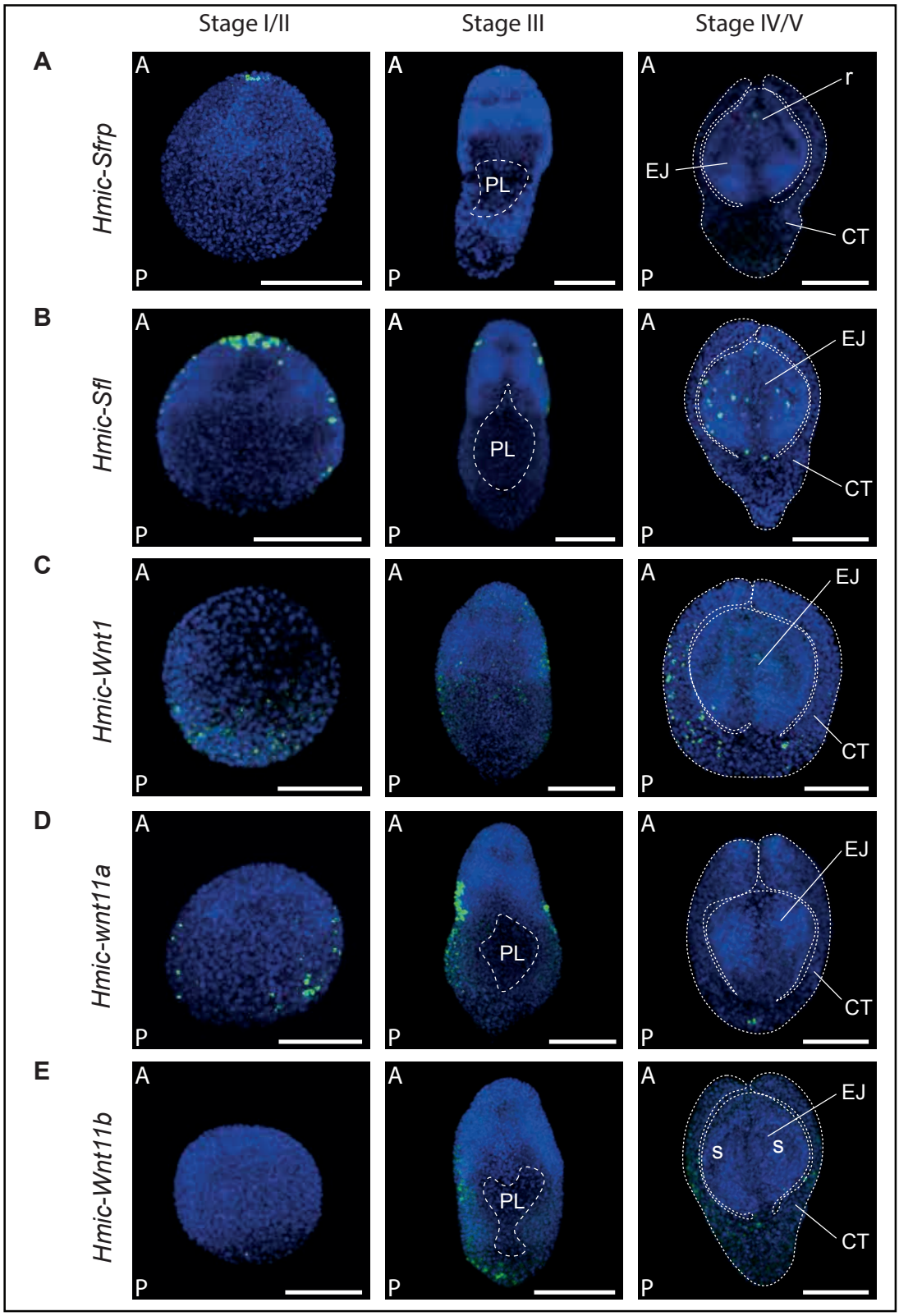

Figure 5.2 Expression of 'posterior' *Wnts* and their antagonists during *Hymenolepis microstoma* larval development. FISH Maximum projections of A) *Hmic-Sfrp*, B) *Hmic-Sfl*, C) *Hmic-Wnt1*, D) *Hmic-Wnt11a* and E) *Hmic-Wnt11b*. Larvae are staged according to Voge (1964). A = anterior pole, CT = cyst tissue, EJ = encysted juvenile worm, P = posterior pole, PL = primary

lacuna, r = rostellum, s = suckers. Whole-mount *in situ* hybridisation signal is shown in green and DAPI in blue. Bars: 50μm

5.2.2.3 Other Wnt factors

The expression of the receptor *Hmic-Fz4/B* is similar to that of *Hmic-Wnt1* - initially diffuse and ubiquitous during early stages, it becomes restricted to the posterior as anterior features develop during metamorphosis (Fig. 5.3 D). *Hmic-Fz4/B* expression is limited to cyst tissue in the cysticercoid (Fig. 5.3 D).

The expression of *Hmic-Wnt2* during the earlier stages of larval development is confined to two anterolateral foci (Fig. 5.3 A). As larvae grow, by stage III, the expression expands into more foci surrounding the base of the early rostellum (Fig. 5.3 A). When development of the cysticercus is completed, *Hmic-Wnt2* remains active around the rostellum (Fig. 5.3 A).

Hmic-Wnt4 is expressed in a central-anterior region during early larval metamorphosis coupled with two lateral nodes (Fig. 5.3 C). During mid-stages of development, this expression remains anteriorised and once metamorphosis is complete, expression is within the scolex of the juvenile worm, especially towards the base (Fig. 5.3 C).

Hmic-Wnt5 expression is observed in double lateral stripes at the onset of larval metamorphosis (Fig. 5.3 B). Lateral expression remains as the worm continues to develop. By the time encystment occurs, *Hmic-Wnt5* is observed laterally within the scolex around the suckers and within the cyst tissue (Fig. 5.3 B).

5.2.3 Adult expression

5.2.3.1 *Wnt* antagonists are expressed towards the anterior of the adult worm during early development

Hmic-Sfrp expression can be categorised into two zones of expression: pre-strobilar and segment maturation. Initially, it is restricted to the neck and scolex (Fig. 5.4 A-B, D). In the scolex, *Hmic-Sfrp* is seen at the base of the rostellum and within the cephalic ganglia (Fig. 5.4 C. Discrete foci are visible throughout the neck and in cross-section, *Hmic-Sfrp* is restricted to the outer cortex (Fig. 5.4 E). *Hmic-Sfrp* appears to be stronger laterally, giving the impression of lateral stripes running down the neck (Fig. 5.4 A). Once the neck ends, *Hmic-Sfrp* expression halts. Once segments have matured, *Hmic-Sfrp* turns on once again, in the ovary, uterus and surrounding the genital pore.

Hmic-Sfl expression is continuous, but is clearly regionalised (Fig. 5.5 A-B), and can be categorised into several discrete zones along the length of the strobila. In the scolex, *Hmic-Sfl* is expressed strongly at the base of the suckers and rostellum in the rostellar ring, possibly at the commissures with the cephalic ganglia. In the neck, *Hmic-Sfl* expression can be subdivided into two zones. Firstly, in roughly the first three-quarters of the neck, in several lateral and medial ribbons of cells in the cortex, and a central stripe through the middle of the worm, in the medulla (Fig. 5.5 B). Secondly, towards the end of the neck, new medial stripes turn on and then all expression begins to

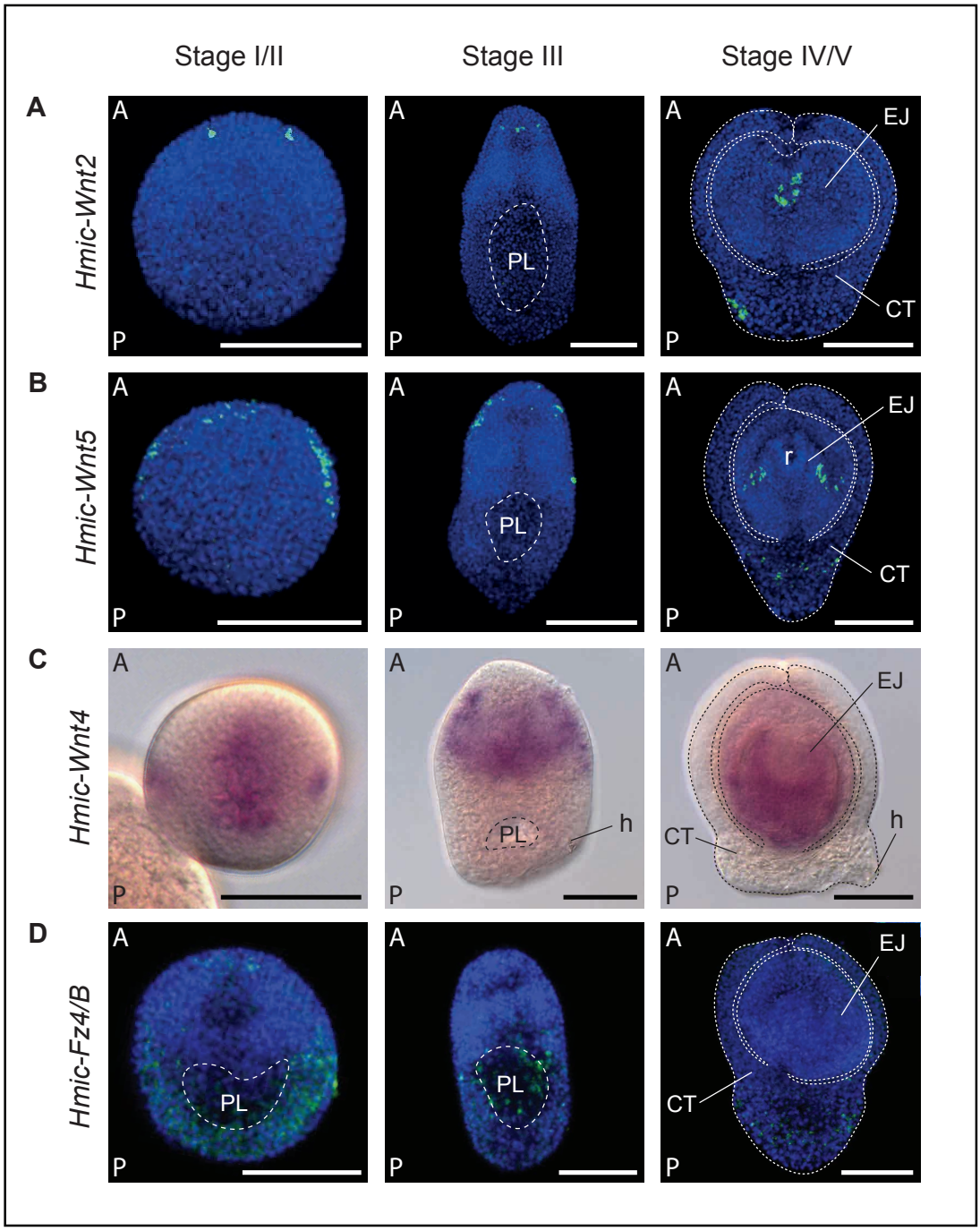

Figure 5.3 Expression of other Wnt genes during *Hymenolepis microstoma* larval development. FISH maximum projections of A) *Hmic-Wnt2*, B) *Hmic-Wnt5*. C) Single plane colorimetric ISH image of *Hmic-Wnt4*. D) FISH maximum projection of *Hmic-Fz4/B*. A = anterior pole, CT = cyst tissue, EJ = encysted juvenile worm, h = hooks, P = posterior pole, PL = primary lacuna. Colorimetric whole-mount *in situ* hybridisation signal is shown in purple, FISH in green and DAPI in blue. Bars: 50μm

fade (Fig. 5.5 B). Beyond the neck, the central and outermost lateral foci turn back on, becoming stronger, and the central node expands (Fig. 5.5 B). As segmentation becomes apparent, a ring of expression is observed along the trailing edge (i.e. anterior) of each segment in a weakening gradient towards the posterior, about a third of the way down into the segment (Fig. 5.5 E). As segments mature, *Hmic-Sfl* is also observed in the developing external and internal seminal vesicles and later in the ovary (Fig. 5.5 F-G).

5.2.3.2 Expression of 'posterior' *Wnts*

Hmic-Wnt1 expression begins late, towards the end of the neck (Fig. 5.6 A). Initially this is in four central loci that appear to be located close to the median nerve cords. As segments begin to form and first become visible a central node of expression appears. Shortly after this, a punctate ladder-like pattern of expression is observed in discrete cells at the leading edge (i.e. the posterior) of each segment. In cross-section, this appears as a ring in the cortex. This ladder-like expression of *Hmic-Wnt1* is continuous, the length of the strobila. As segments mature, the centralised expression of *Hmic-Wnt1* continues and is eventually observed in the ovary and external seminal vesicle. Once proglottides have matured, *Hmic-Wnt1* is observed in the uterus. *Hmic-Wnt11a* is expressed first in a central, continuous, stripe towards the end of the neck and then two lateral stripes (Fig. 5.7 A-B). Gradually, at the point where the neck begins to transition into the rest of the strobila, a ring of stripes or 'ladder' pattern forms (Fig. 5.7 A-B). Once this 'ladder' is established the central stripe fades, eventually stopping as the

ladder continues. In cross-section, the ‘ladder’ of *Hmic-Wnt11a* is expressed in two concentric rings in the cortex (Fig. 5.7 C). Once the early segments are visible, *Hmic-Wnt11a* expression fades with the ‘ladder’ disappearing with the lateral most parts being the last to stop completely. In mature segments, *Hmic-Wnt11a* turns back on and is observed within the ovary and uterus (Fig. 5.7 D-F). *Hmic-Wnt11b* turns on post neck and is observed in faint stripes along the leading edge of segments and in the seminal receptacle (Fig. 5.6).

5.2.3.3 Expression of *Frizzled* receptors

Wnt receptors are all expressed in a similar manner in the cortex (Figs. 5.8-10). In the *Hmic-Fz*s studied, (*Hmic-FzA*, *Hmic-Fz4/B*, *Hmic-FzD* and *Hmic-FzE*), this appears as punctate expression close to the surface of the worm (Figs. 5.8-10). In cross section, this expression forms a ring, just beneath the tegument and is consistent between all four *Hmic-Fz*s (Figs. 5.8-10). Additional patterns for each *Hmic-Fz* alongside this cortical expression are also observed. *Hmic-FzA* is also expressed in cells within the outer muscle layer of the scolex (Fig. 5.8). *Hmic-Fz4/B*, *Hmic-FzD* and *Hmic-FzE* are all expressed in a central stripe in the neck that is likely to represent the genital primordium (Figs. 5.9-10). This centralised expression continues in *Hmic-Fz4/B*, which, in immature segments, is observed in the seminal receptacle and in mature segments, the genital pore (Fig. 5.9). A small amount of *Hmic-FzE* expression is observed in the medulla (Fig. 5.10 E).

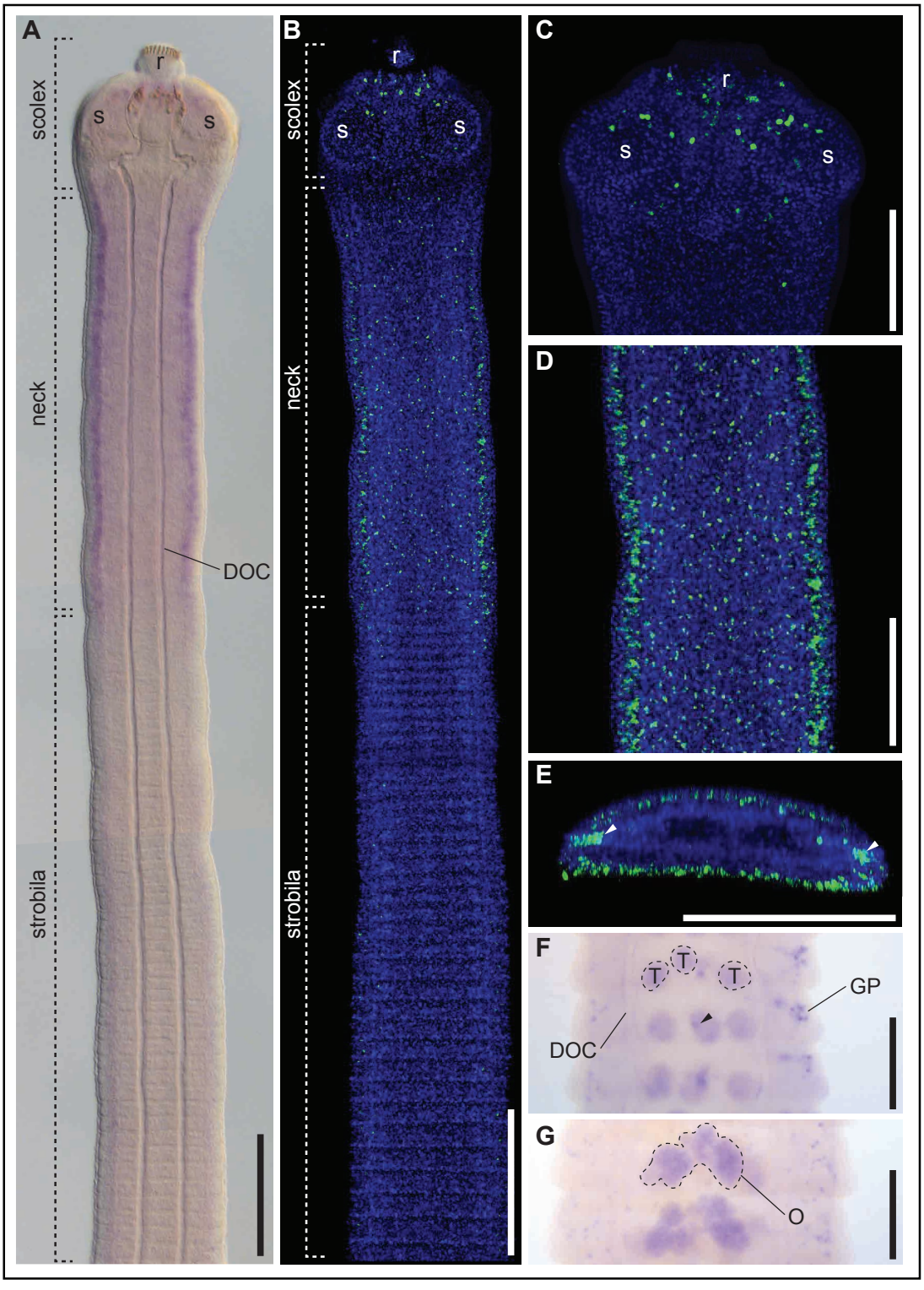

Figure 5.4 Expression of *Hmic-Sfrp* in adult *Hymenolepis microstoma*. A) Colorimetric ISH and B) FISH of the adult worm. C) Expression within the scolex, D) neck and E) cross section of the neck. Arrowheads highlight lateral expression of *Hmic-Sfrp*. Colorimetric ISH in mature segments highlighting expression in F) ovary and genital pore (arrowhead indicates central expression during early development of the ovary) and G) uterus. DOC = dorsal osmoregulatory canal, GP = genital pore, O = ovary, r =

rostellum, rb = rostellar bulb, s = suckers, T = testes. Colorimetric whole-mount *in situ* hybridisation signal is shown in purple, FISH in green and DAPI in blue. Bars: 100μm

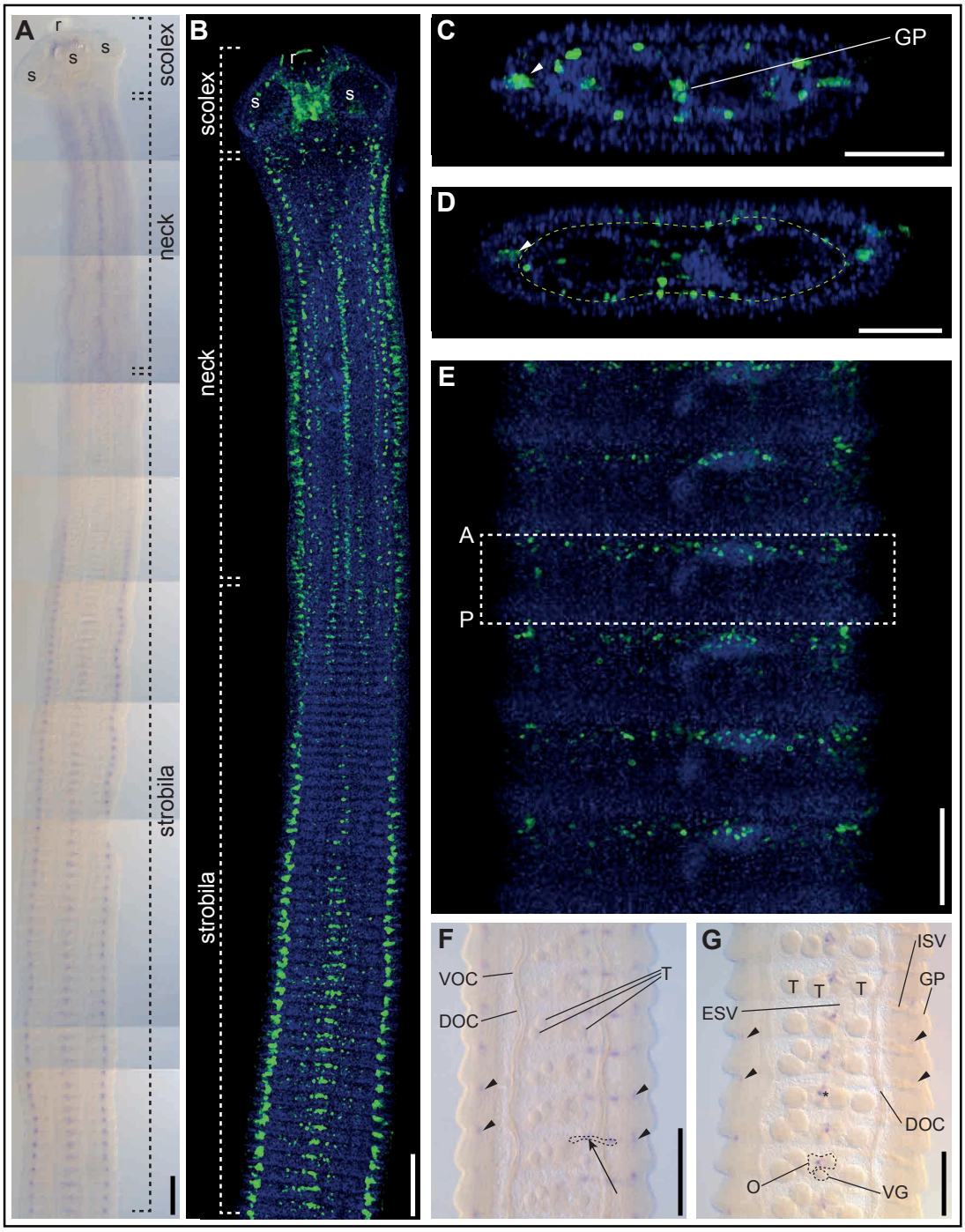

Figure 5.5 Expression of *Hmic-Sfl* in adult *Hymenolepis microstoma*. A) Colorimetric ISH and B) maximum projection FISH of the adult worm. Expression is visible in the scolex, and in the neck in a central and several medial and several lateral stripes. Around the border between the neck and strobila, expression patterns begin to change. In the strobila, the central node of expression expands as segments mature and the lateral foci turn back on. Cross sections of C) the neck and D) immature segments. Dashed green line indicates the position of a transverse nerve cord, white arrowheads indicate a lateral cluster of cells that run the length of the adult worm. E) Polarised expression in segments, dashed box designates one

segment. Colorimetric ISH in mature segments highlighting expression in F) seminal receptacle and G) ovary. Black arrowheads highlight lateral nodes of expression, arrow indicates the seminal receptacle and an asterisk shows expression within the centre of the ovary. A = anterior, DOC = dorsal osmoregulatory canal, ESV = external seminal vesicle, GP = genital primordia, ISV = internal seminal vesicle, O = ovary, P= posterior, r = rostellum, s = suckers, T = testes, VG = vitelline gland, VOC = ventral osmoregulatory canal. Colorimetric whole-mount *in situ* hybridisation signal is shown in purple, FISH in green and DAPI in blue. Bars: 100μm

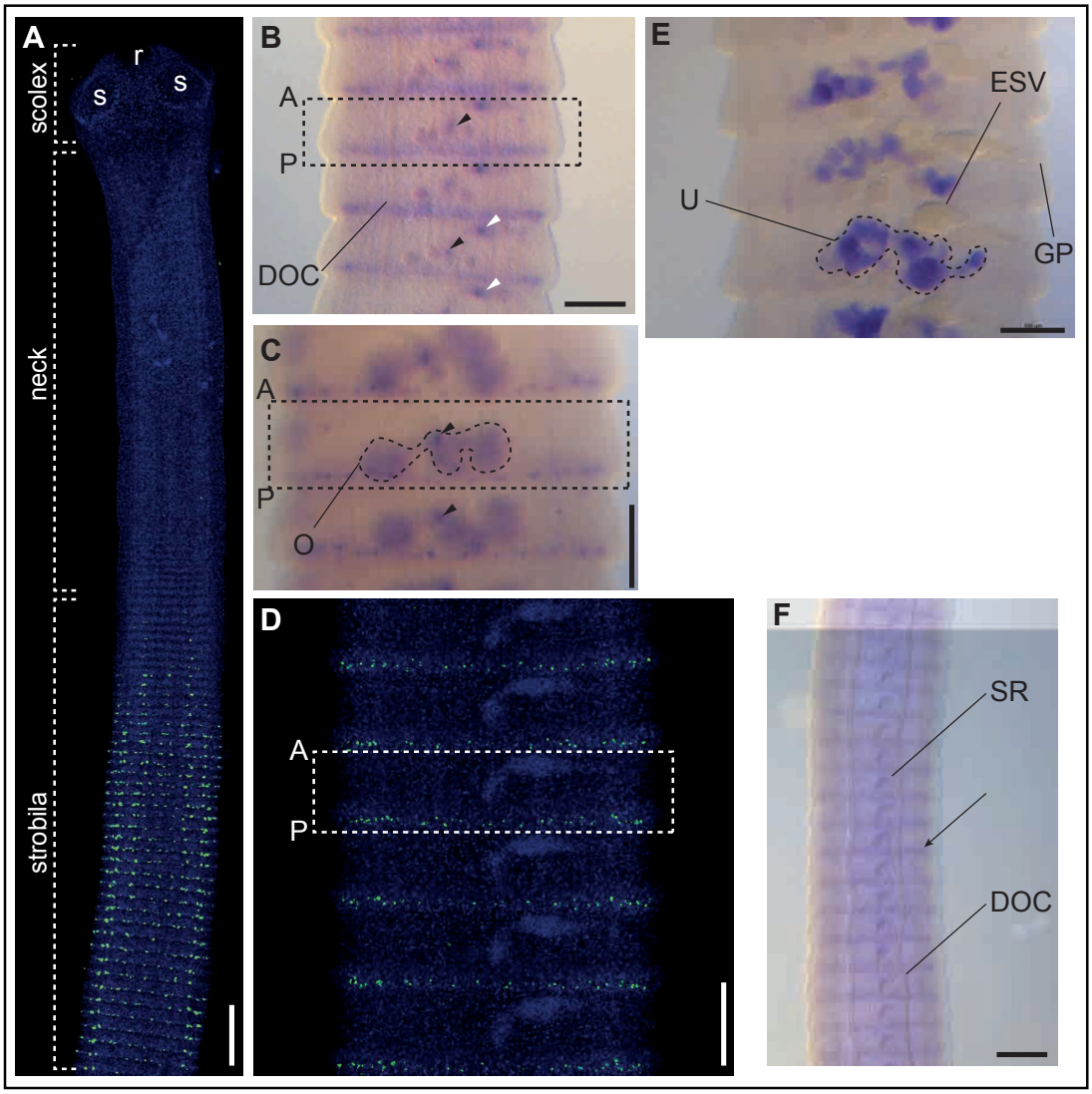

Figure 5.6 Expression of *Hmic-Wnt1* and *Hmic-Wnt11b* in adult *Hymenolepis microstoma*. A) FISH of *Hmic-Wnt1* in the adult worm showing expression turns on after the neck. Colorimetric ISH of B) immature segments showing polarised, posterior expression within each segment, expression within the developing ovary (black arrowheads) and expression within the external seminal vesicle (white arrowheads), C) mature segments showing expression in the ovary, where stronger expression is observed in the centre of the ovary (black arrowhead). D) FISH of *Hmic-Wnt1* in segments showing polarised and punctate expression. Colorimetric expression of *Hmic-Wnt11b* in E) mature segments in the uterus and F) immature segments. Arrows point to faint polarised, posterior expression within segments. Dashed boxes indicate one segment. A = anterior, DOC = dorsal osmoregulatory canal, ESV = external seminal vesicle, GP = genital pore, P = posterior, r = rostellum, s= suckers, SR = seminal receptacle, U = uterus. Colorimetric whole-mount *in situ* hybridisation signal is shown in purple, FISH in green and DAPI in blue. Bars: 100μm

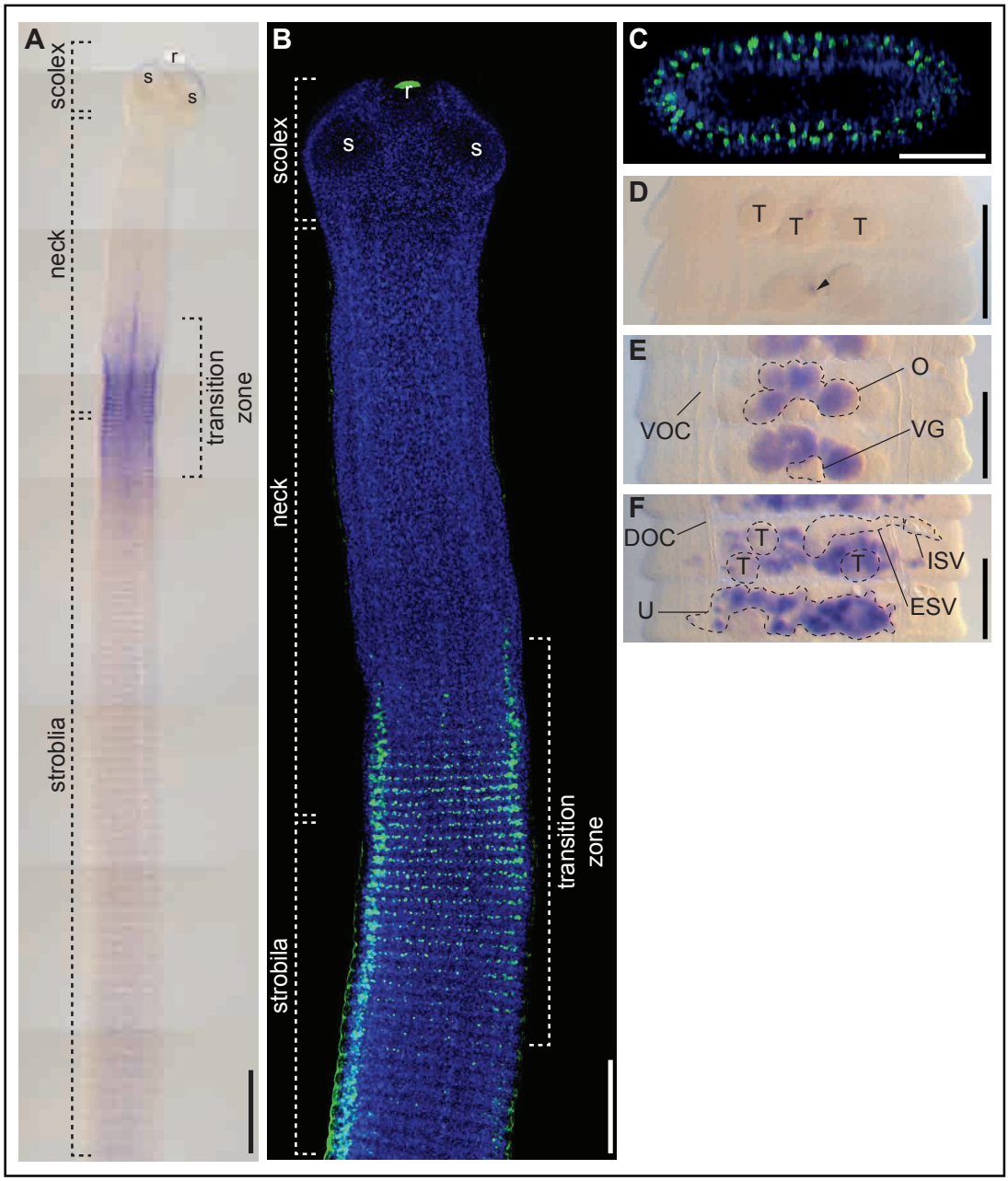

Figure 5.7 Expression of *Hmic-Wnt11a* in adult *Hymenolepis microstoma*. A) Colorimetric ISH and B) maximum projection FISH of *Hmic-Wnt11a* in the adult worm showing expression exclusively within the 'transition zone'. C) Cross section through the 'transition zone' shows double rings of expression. Colorimetric ISH in mature segments showing expression in D) the centre of the immature ovary (indicated by arrowhead), E) mature ovary and F) uterus. Colorimetric expression of *Hmic-Wnt11b* in E) mature segments in the uterus and F) immature segments. DOC = dorsal osmoregulatory canal, ESV = external seminal vesicle, ISV = internal seminal vesicle, O = ovary, r = rostellum, s = suckers, T = testes, U = uterus, VOC = ventral osmoregulatory canal, VG = vitelline gland. Colorimetric whole-mount *in situ* hybridisation signal is shown in purple, FISH in green and DAPI in blue. Bars: 100μm

Unfortunately, *Hmic-FzC* could not be amplified and, due to time restrictions, the other remaining *Hmic-Fz*s could not be analysed. These should be studied next in future investigations.

5.2.3.4 Expression of other *Wnts*

The expression of non-canonical *Wnts* appear to be associated with the maturation of the reproductive system and early embryogenesis. In mature segments, *Hmic-Wnt2* is diffuse, but stronger in the testes, ovaries and uterus (Fig. 5.11 G-H). *Hmic-Wnt4* expression in the adult is found in maturing segments in the testes, ovaries, vitellaria, seminal receptacle and along the vagina (Fig. 5.11 C-F). Later, in more mature segments, it can be seen surrounding the genital pore and in developing embryos within the uterus. This expression is mirrored in *Hmic-Wnt5* which is expressed in the testes, ovary, genital pore and uterus (Fig. 5.12). A second theme of non-canonical *wnt* expression is also observed in both *Hmic-Wnt4* and *Hmic-Wnt5* – this is in cells in the outer cortex, close to the tegument (Figs. 5.11 A and 5.12 A) In cross section, this appears as a ring (Figs. 5.11 B and 5.12 B) and mirrors the cortical expression of *Hmic-Fz*s (Figs. 5.8-9). It is most likely that expression is in muscle underneath the tegument.

5.2.4 Pre-strobilar expression

Hmic-Wnt1 expression in newly excysted juveniles is restricted to a few cells in the posterior most part of the worm (Fig. 5.13 A). As the juvenile worm

grows, *Hmic-Wnt1* continues to be constrained to the posterior, with no expression in the scolex. At the onset of strobilation, *Hmic-Wnt1* remains activated in the posterior of the worm (Fig. 5.13 C). *Hmic-Wnt11a*, like *Hmic-Wnt1*, in 24 hr old juveniles is limited to the posterior of the worm, but is more extensive, with expression observed in approximately the posterior third of the excysted juvenile (Fig. 5.13 B). In worms just beginning to strobilate, *Hmic-Wnt11a* is still in the posterior, in a punctate, ladder-like pattern where segments are starting to form (Fig. 5.13 D-E). At this stage, each 'rung' of *Hmic-Wnt11a* expression represents each nascent segment as it is forming. As in adults, when looked at in cross-section, this expression is in a ring in the cortex. Once strobilation is fully established, expression mirrors the pattern observed in adults, and is expressed in roughly 30 nascent segments.

Despite multiple attempts, expression patterns of the anterior markers *Hmic-Sfrp* and *Hmic-Sfl* in pre-strobilar worms could not be achieved. This was most likely due to the quality of juvenile material which quickly degraded after recovery from infected mice.

5.2.5 A close association between Wnt factors and the nervous system

The expression patterns of some Wnt factors would suggest that they are expressed close to the median nerve cords and other major nerve junctions. To test this, co-expression analysis using the immunohistochemical marker synapsin with *Hmic-Wnt1* and *Hmic-Sfrp* were carried out.

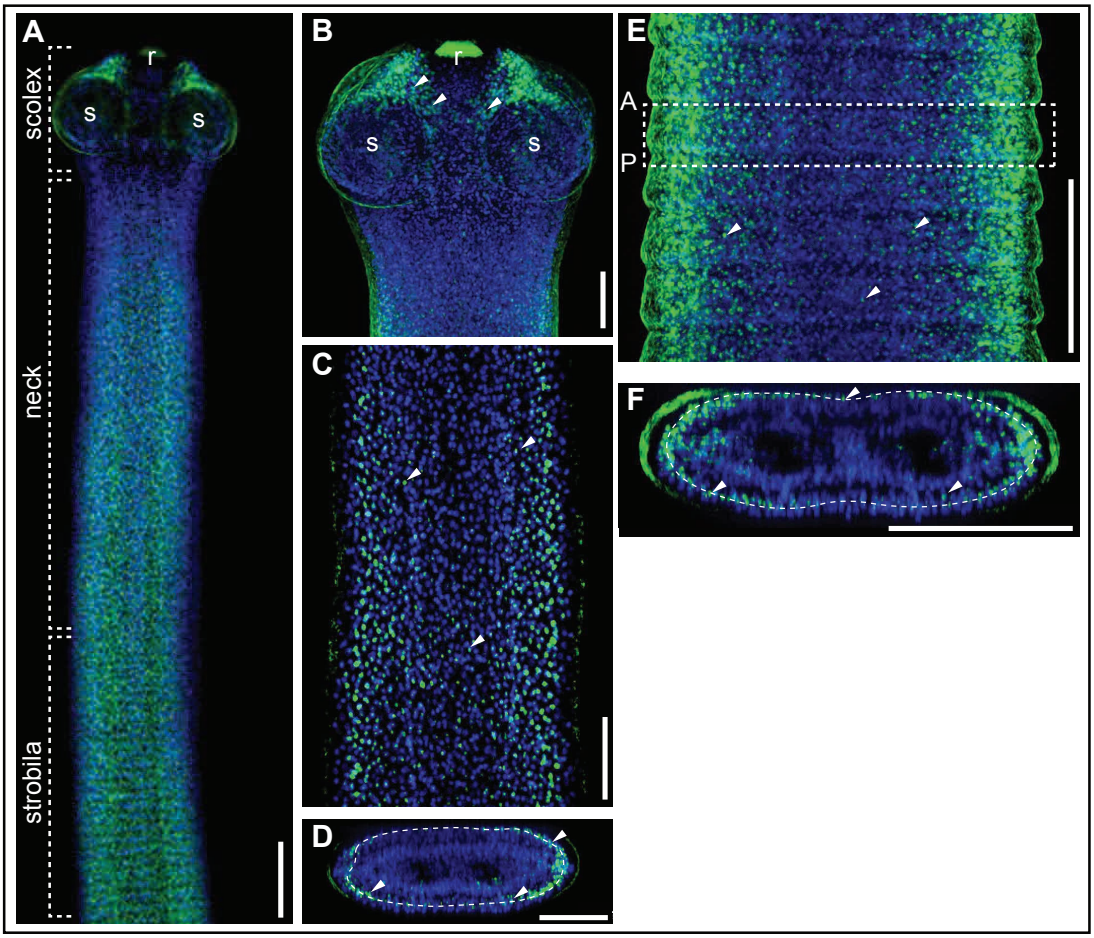

Figure 5.8 Expression of *Hmic-FzA* in adult *Hymenolepis microstoma*. FISH of A) adult worm, B) scolex, C) neck, D) cross section of the neck, E) immature segments, where the box designates one segment and F) cross section of immature segments. Expression is observed to be punctate (highlighted by arrowheads) and is restricted to the cortex (dashed ring). A = anterior, P = posterior, r = rostellum, s = suckers Whole-mount *in situ* hybridisation signal is shown in green and DAPI in blue. Bars: 100μm

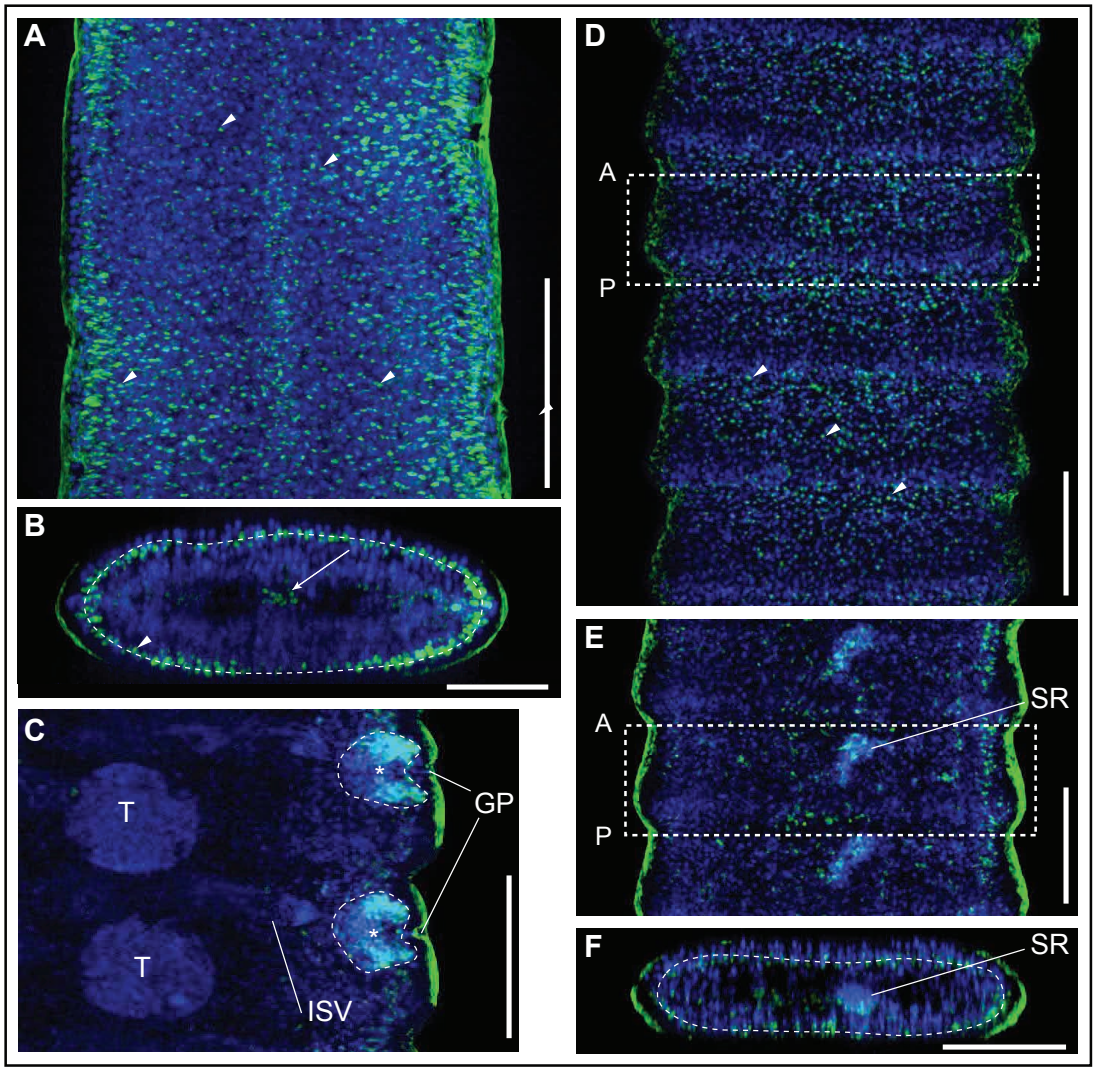

Figure 5.9 Expression of *Hmic-Fz4/B* in adult *Hymenolepis microstoma*. FISH of A) neck, B) cross section of neck, expression is punctate (highlighted by arrowheads) restricted to the cortex (dashed ring) and also a central stripe of expression within the genital primordium (arrow). C) Expression in mature segments shows expression around the genital pore (asterisk). Expression in immature segments shows D) continued punctate expression in the cortex and E) centralised expression in the seminal receptacle. F) Expression of within the cortex (indicated by dahed ring) and seminal receptacle is observed more clearly in a cross section of immature segments. Boxes designate one segment. A = anterior, ISV = internal seminal vesicle, GP = genital pore, P = posterior, SR = seminal receptacle, T = testes. Whole-mount *in situ* hybridisation signal is shown in green and DAPI in blue. Bars: 100μm

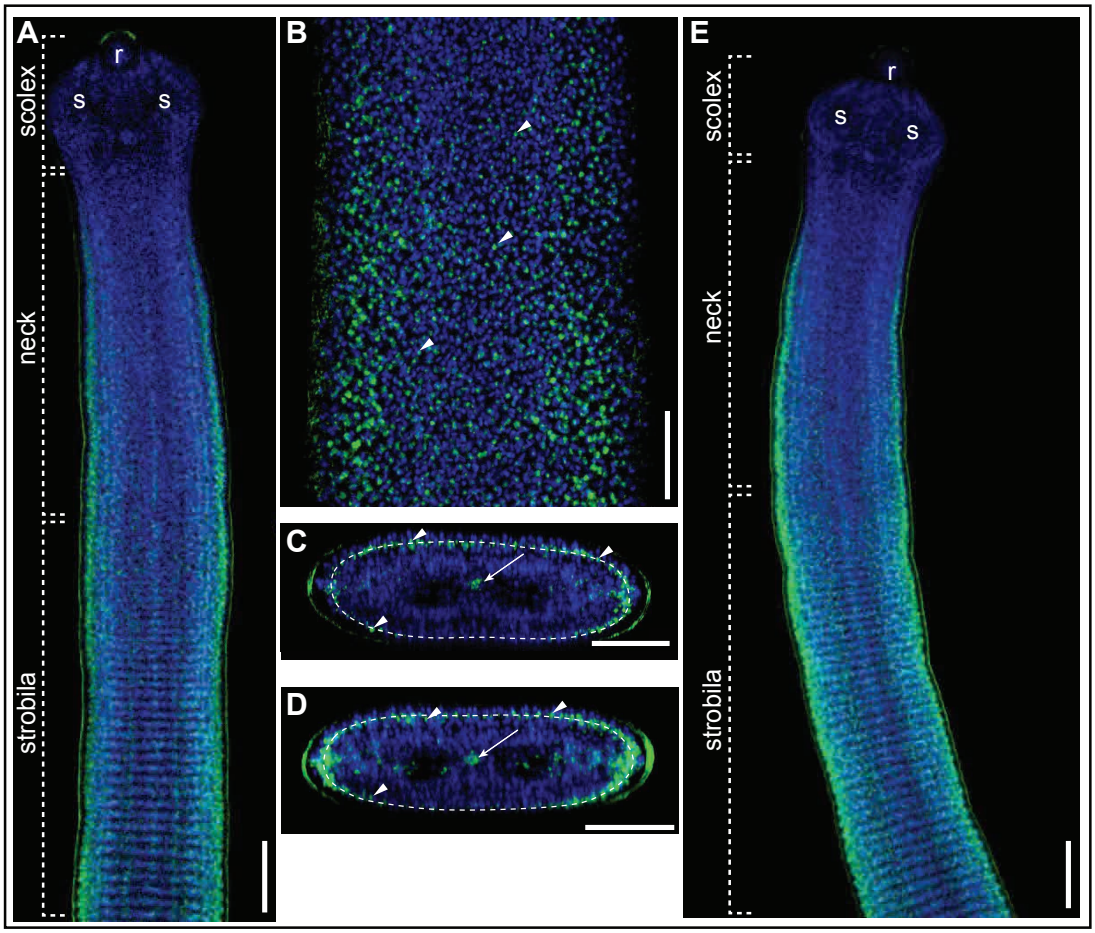

Figure 5.10 Expression of *Hmic-FzD* and *Hmic-FzE* in adult *Hymenolepis microstoma*. FISH of *Hmic-FzD* in A) adult worm, B) neck and C) cross section of neck. Punctate expression (indicated by arrowheads) is found in the cortex (dashed ring) and is also found in the genital primorida (arrow). FISH of *Hmic-FzE* in D) cross section of the neck and E) the adult worm. Expression is observed in the cortex (dashed ring) and genital primordia (arrow). r = rostellum, s = suckers. Whole-mount *in situ* hybridisation signal is shown in green and DAPI in blue. Bars: 100μm

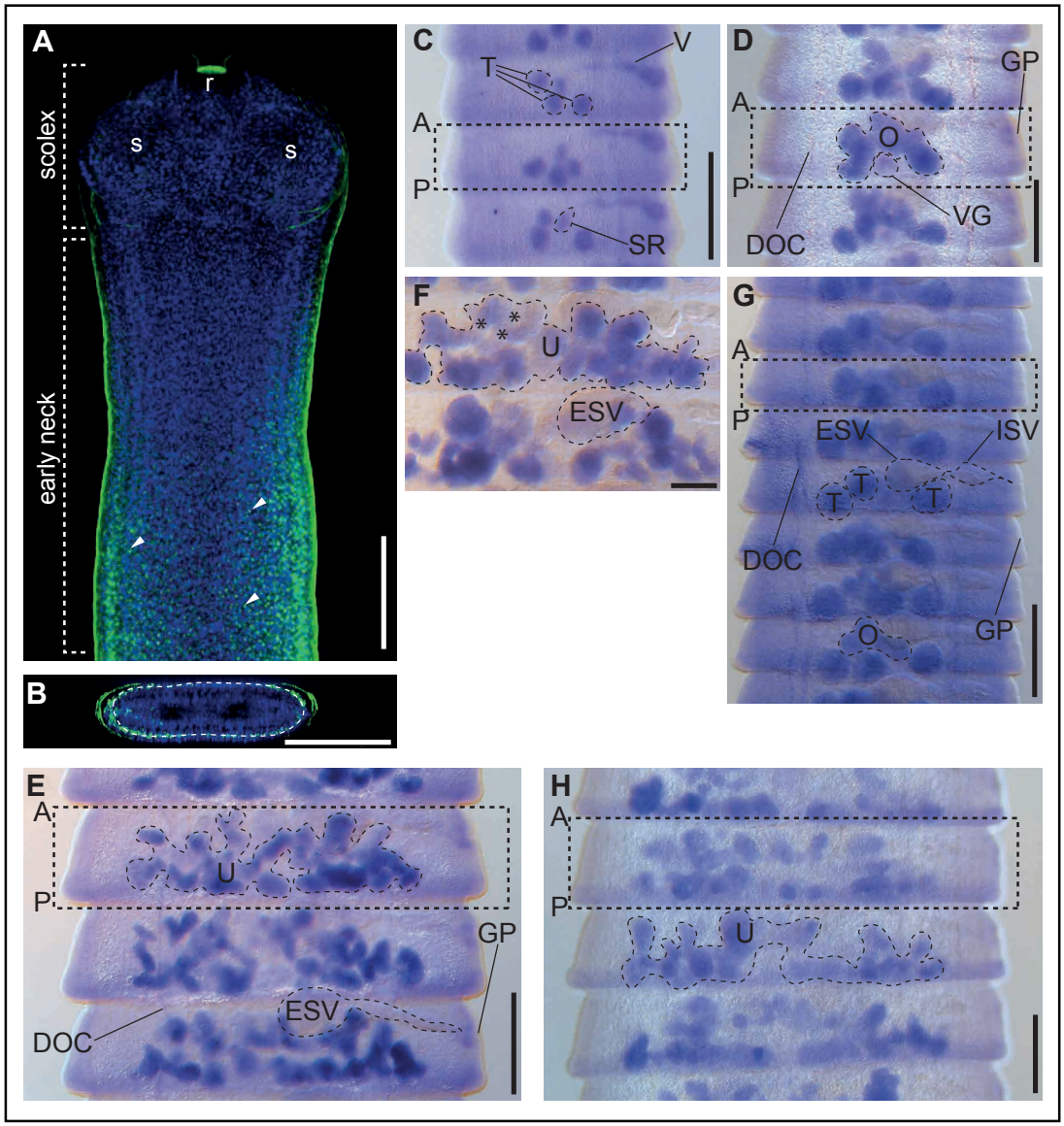

Figure 5.11 Expression of *Hmic-Wnt4* and *Hmic-Wnt2* in adult *Hymenolepis microstoma*. FISH of *Hmic-Wnt4* in A) the scolex and neck and B) cross section of the neck. In the neck, expression is punctate (arrowheads) and is restricted to the cortex. Colorimetric ISH of *Hmic-Wnt4* in C) immature segments expression can be seen in the testes, seminal receptacle and along the vagina. In D) mature segments expression is in the ovary and vitelline gland. Later, in E) more mature segments, expression is within the uterus. F) In the uterus, expression can be seen in developing embryos (indicated by asterisk). Colorimetric ISH of *Hmic-Wnt2* in G) mature segments shows expression in the testes and ovary whilst in H) more mature segments, expression is observed in the uterus. Dashed boxes designate one segment. A = anterior, DOC = dorsal osmoregulatory canal, ESV = external seminal vesicle, ISV = internal seminal vesicle, GP = genital pore, O = ovary, P = posterior, r = rostellum, s = suckers, SR = seminal receptacle, T = testes, U = uterus, V = vagina, VG = vitelline gland. Colorimetric whole-

mount *in situ* hybridisation signal is shown in purple, FISH in green and DAPI in blue. Bars: 100μm

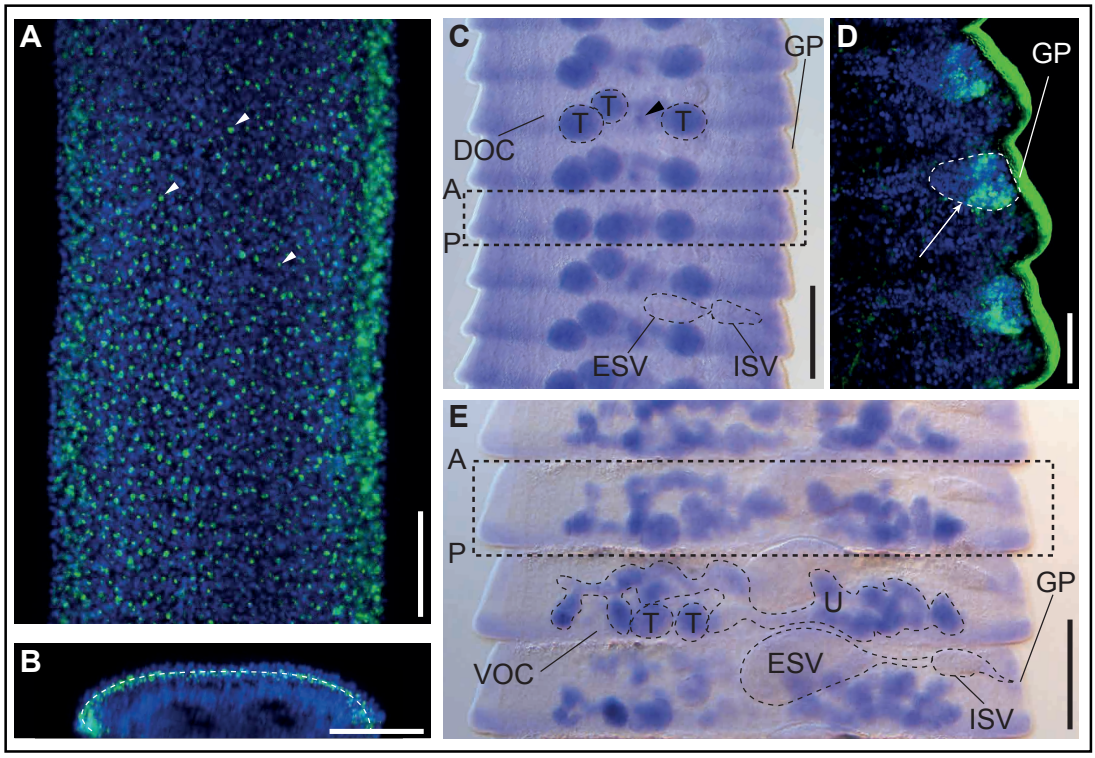

Figure 5.12 Expression of *Hmic-Wnt5* in adult *Hymenolepis microstoma*. FISH in A) the neck B) cross section of the neck. In the neck, expression is punctate (white arrowheads) and restricted to the cortex (dashed ring). Colorimetric ISH in C) mature segments reveals expression in the testes. A foci of expression is also observed in the centre of the ovary (black arrowhead). D) FISH in mature segments showing expression around the genital pore (indicated by arrow). E) In more mature segments, expression is within the uterus. Dashed boxes designate one segment. A = anterior, DOC = dorsal osmoregulatory canal, ESV = external seminal vesicle, ISV = internal seminal vesicle, GP = genital pore, P = posterior, T = testes, U = uterus, VOC = ventral osmoregulatory canal. Colorimetric whole-mount *in situ* hybridisation signal is shown in purple, FISH in green and DAPI in blue. Bars: 100μm

Figure 5.13 Expression of *Hmic-Wnt1* and *Hmic-Wnt11a* in pre-strobilar and early strobilating *Hymenolepis microstoma*. A) *Hmic-Wnt1* and B) *Hmic-Wnt11a* in pre-strobilar worms. Expression of both *Hmic-Wnt1* and *Hmic-Wnt11a* is posteriorised, indicated by arrows. C) Expression of *Hmic-Wnt1* prior to the onset of strobilation. Prior to strobilation, punctate expression (indicated by arrowheads) is observed in the posterior of the worm. *Hmic-Wnt11a* expression at D) the onset of strobilation and E) once strobilation is established. Here, expression is restricted to the posterior in stripes along nascent and early segments. r = rostellum, s = suckers, zs = zone of strobilation. Whole-mount *in situ* hybridisation signal is shown in green and DAPI in blue. Bars: 50μm

Early *Hmic-Wnt1* expression towards the end of the neck is limited to four nodes of expression (Fig. 5.6). Co-expression with synapsin shows that there is a close association between cells expressing *Hmic-Wnt1* and synapsin (Fig. 5.14). In many sections, *Hmic-Wnt1* is observed to be nested next to the median nerve cords (Fig. 5.14 F-G). The expression of *Hmic-Wnt1* in relation to the nerve cord is not consistent, however. In some cases, in both the late neck and strobila, *Hmic-Wnt1* expression is observed centrally of the commissure between the median and main transverse nerve cords whilst in others, it is observed more externally 'outside' the commissure. In a few sections, co-localisation is observed. In immature and mature segments, the close relationship between *Hmic-Wnt1* and the nervous system is much clearer. Here, larger nodes of *Hmic-Wnt1* expression appear to be associated with the lateral and median nerve cords, in some cases, co-localising (Fig. 5.14 A-B). The posterior punctate ring of *Hmic-Wnt1* expression seen in every segment is located along the major transverse nerve of each segment (Fig. 5.14 B).

Both *Hmic-Sfrp* expression and staining against synapsin are observed within the scolex and are found to co-localise (Fig. 5.15). Several strong nodes of *Hmic-Sfrp* are expressed and these are associated with major nerve junctions of the brain, in the cerebral ganglia (Fig. 5.15 A-B). Further co-localisation is observed towards the nerve at the base of the rostellar ring and in innervations to the suckers. Not all *Hmic-Sfrp* expression within the scolex co-localises with synapsin – some foci are observed on their own in the suckers and rostellum. In the neck, other than a few nodes of *Hmic-Sfrp*

expression associated with the longitudinal nerve cords, *Hmic-Sfrp* does not co-localise with synapsin (Fig. 5.15 C).

5.2.6 Co-expression of Wnt factors

5.2.6.1 Early co-localisation of *Hmic-Sfl* and *Hmic-Wnt1*

Double FISH (dFISH) of *Hmic-Sfl* and *Hmic-Wnt1* shows that as the expression of *Hmic-Sfl* changes towards the end of the neck, *Hmic-Wnt1* is turned on (Fig. 5.16 A). Initially, both *Hmic-Sfl* and *Hmic-Wnt1* are co-expressed in four nodes that are associated with the median nerves (Fig. 5.15 C-E). As strobilation continues and segments start to become visible, the co-localisation of these two factors stops. The nodes separate out and as early segments appear, two distinct rings of expression are visible. As segments mature it becomes clear that each ring of expression is polarised within each segment – *Hmic-Sfl* anteriorly and *Hmic-Wnt1* posteriorly (Fig. 5.16 G-H). This polarised expression is clearest in mature segments where gene expression remains on (Fig. 5.16 H).

5.2.6.2 *Hmic-Wnt11a* and *Hmic-Sfl*

Towards the end of the neck, *Hmic-Sfl* expression begins to change. Initially observed in multiple stripes, it becomes restricted and is only found laterally and centrally. Soon after, four median stripes of expression turn on. dFISH shows that as *Hmic-Sfl* expression changes, *Hmic-Wnt11a* first co-localises

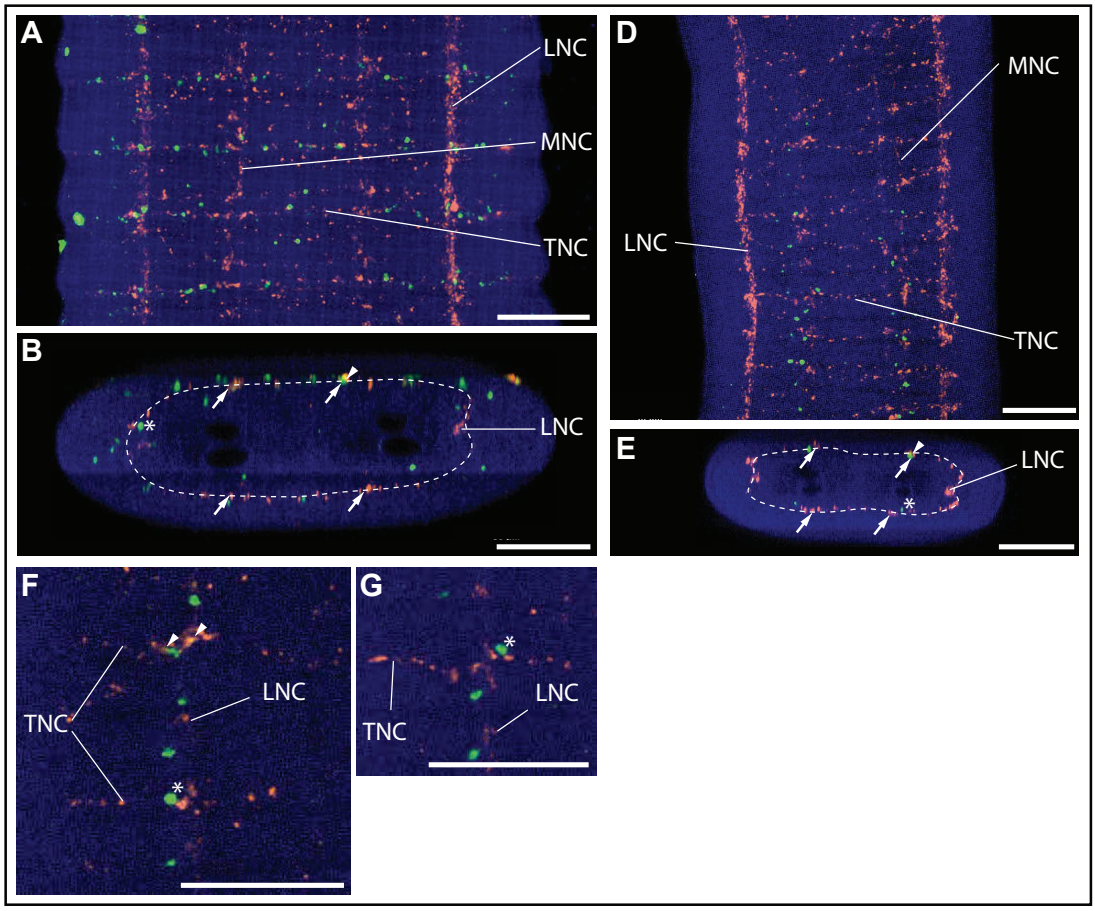

Figure 5.14 Expression of *Hmic-Wnt1* is associated with the nervous system in adult *Hymenolepis microstoma*. Maximum projections showing expression of *Hmic-Wnt1* and immunostaining against synapsin in A) mature segments and B) mature segments in cross section, D) the neck and E) cross section of the neck. The dashed lines indicate the positioning of the transverse nerve cords in cross section that expression of *Hmic-Wnt1* clearly follows in B). Arrows point to the four commissures between the lateral and transverse nerve cords whilst arrowheads highlight co-expression of *Hmic-Wnt1* and synapsin. Asterisks show where expression of *Hmic-Wnt1* occurs next to, but not co-localising with, the nervous system. F) Single plane image of the commissures between the transverse and lateral nerve cords and G) again, under high power (x60). Arrowheads show co-expression of *Hmic-Wnt1* and synapsin whilst asterisks point to where expression of *Hmic-Wnt1* occurs next to, but not co-localising with, the nervous system. LNC = lateral nerve cords, MNC = median nerve cords, TNC = transverse nerve cords. Whole-mount *in situ* hybridisation signal is shown in green, immunostaining of synapsin in pink and DAPI in blue. Bars: 100 μm

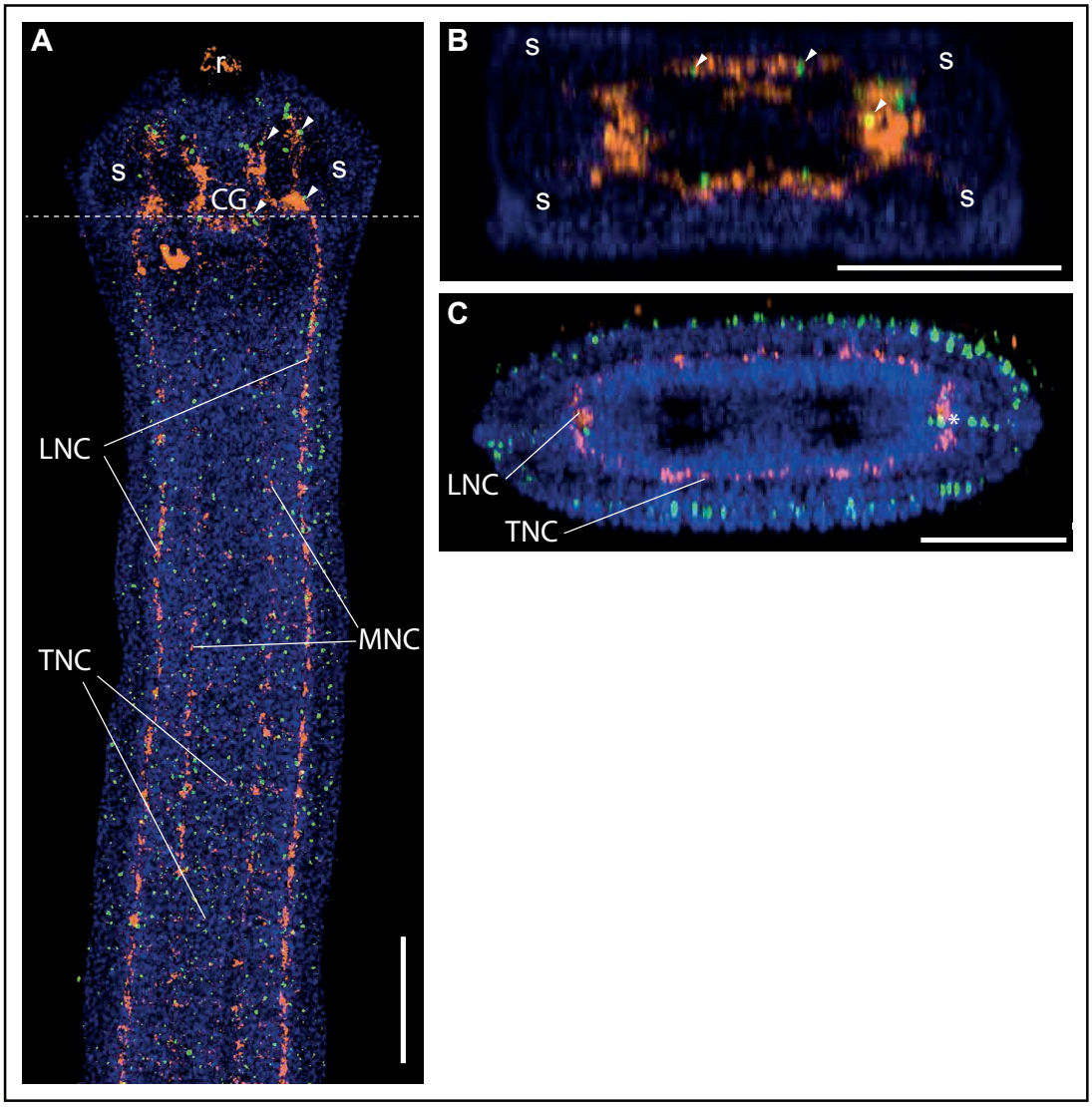

Figure 5.15 Expression of *Hmic-Sfrp* is associated with the nervous system in adult *Hymenolepis microstoma*. Maximum projection of *Hmic-Sfrp* expression and immunostaining against synapsin in A) the neck and scolex. Dashed line indicates B) cross section through the scolex. Arrowheads highlight co-localisation observed within the cephalic ganglia. C) Cross section through the neck. CG = cephalic ganglia, LNC = lateral nerve cords, MNC = median nerve cords, r = rostellum, s = suckers, TNC = transverse nerve cords. Whole-mount *in situ* hybridisation signal is shown in green, immunostaining of synapsin in orange and DAPI in blue. Bars: 100 μm

and then seems to ‘take over’ the lateral and central stripes of *Hmic-Sfl* expression (Fig. 5.17 D-E). Shortly after this change, *Hmic-Wnt11a* begins to form the ladder-like rings of expression. It is only once this pattern of *Hmic-Wnt11a* is established, that *Hmic-Sfl* expression expands, also forming a ladder-like pattern of expression (Fig. 5.17 D). When this expression pattern of *Hmic-Sfl* has become fixed, *Hmic-Wnt11a* turns off (Fig. 5.17).

5.2.6.3 *Hmic-Wnt1* and *Hmic-Wnt11a*

Double staining of *Hmic-Wnt1* and *Hmic-Wnt11a* indicates that *Hmic-Wnt1* expression changes once *Hmic-Wnt11a* is activated (Fig. 5.17 A). Prior to *Hmic-Wnt11a* activation, *Hmic-Wnt1* is observed in four nodes that are repeated along the neck, appearing as stripes, in association with the median nerves. Once the ladder-like pattern of *Hmic-Wnt11a* is established, the expression pattern of *Hmic-Wnt1* expands. From this point onwards, *Hmic-Wnt1* is also observed in punctate, ladder-like rings of expression, but does not co-localise with *Hmic-Wnt11a* (Fig. 5.17 A). *Hmic-Wnt1* remains activated in this pattern after *Hmic-Wnt11a* expression ends.

5.2.6.4 Co-localisation of *Hmic-Sfl* and *Hmic-Hedgehog*?

In early stages of larval development, *Hmic-Sfl* and *Hmic-Hedgehog* (*Hmic-Hh*) are expressed in two opposing stripes that run from the anterior apex of the larva towards the posterior. Later images of larval development show *Hmic-Sfl* is expressed laterally whilst *Hmic-Hh* expression is centralised.

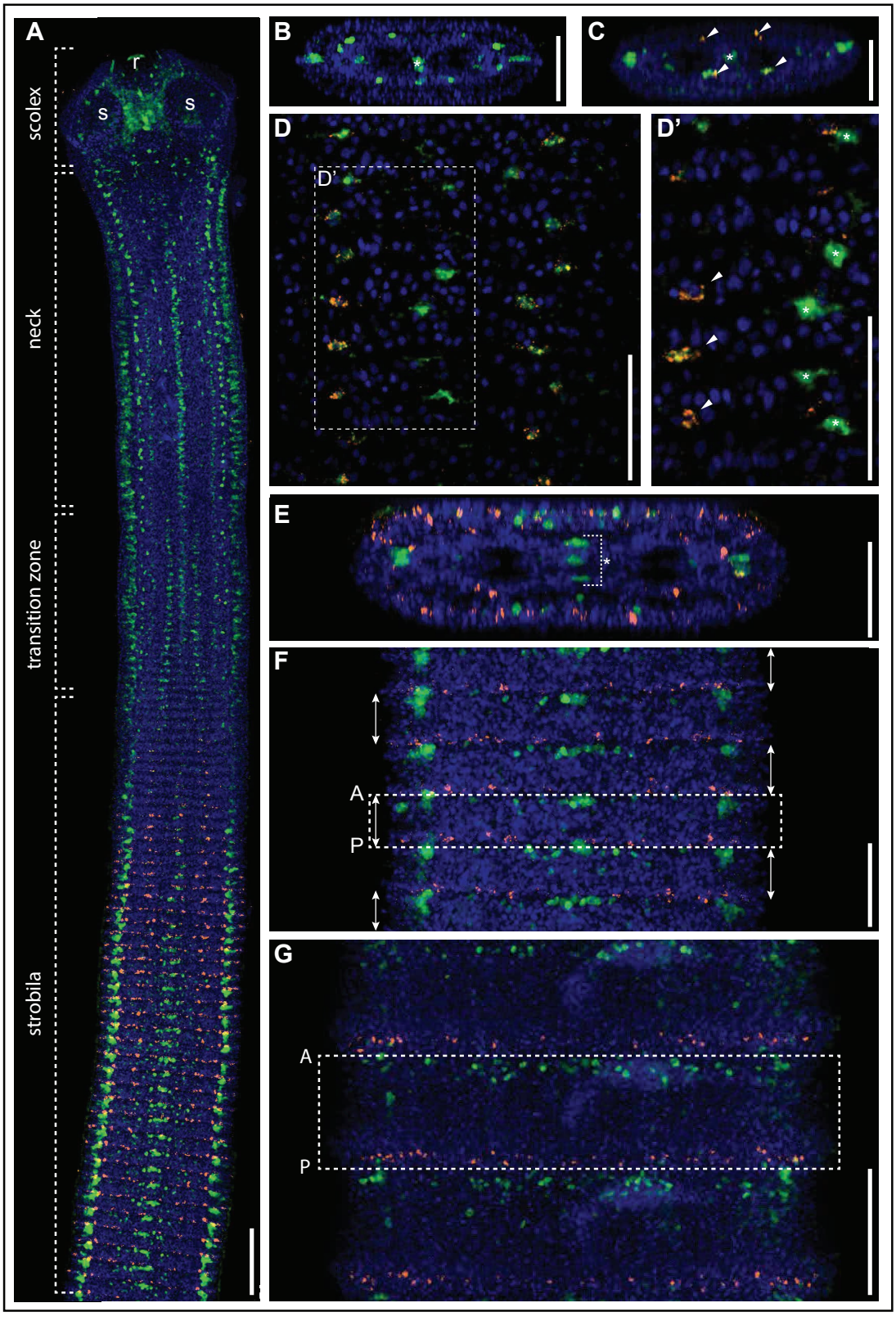

Figure 5.16 Co-expression of *Hmic-Sfl* and *Hmic-Wnt1* in adult *Hymenolepis microstoma*. A) Co-expression in the adult worm. Cross sections of B) the early neck and C) late neck. In the early neck, only *Hmic-Sfl* is expressed, in the late neck, *Hmic-Wnt1* is activated in foci close to the median nerve cords (arrowheads). Co-localisation is observed in D) the

neck. And in D') this co-localisation is clearly seen (arrowheads) whilst in the genital pore (asterisk) only *Hmic-Sfl* is expressed. E) Cross section through early segments. In F) immature and G) mature segments, expression of both *Hmic-Sfl* and *Hmic-Wnt1* becomes polarised. The dashed boxes designate one segment where *Hmic-Sfl* is expressed anteriorly and *Hmic-Wnt1* is expressed posteriorly. *Hmic-Sfl* signal is shown in green, *Hmic-Wnt1* in orange and DAPI in blue. A = anterior, P = posterior, r = rostellum, s = suckers. Bars: 100 μm

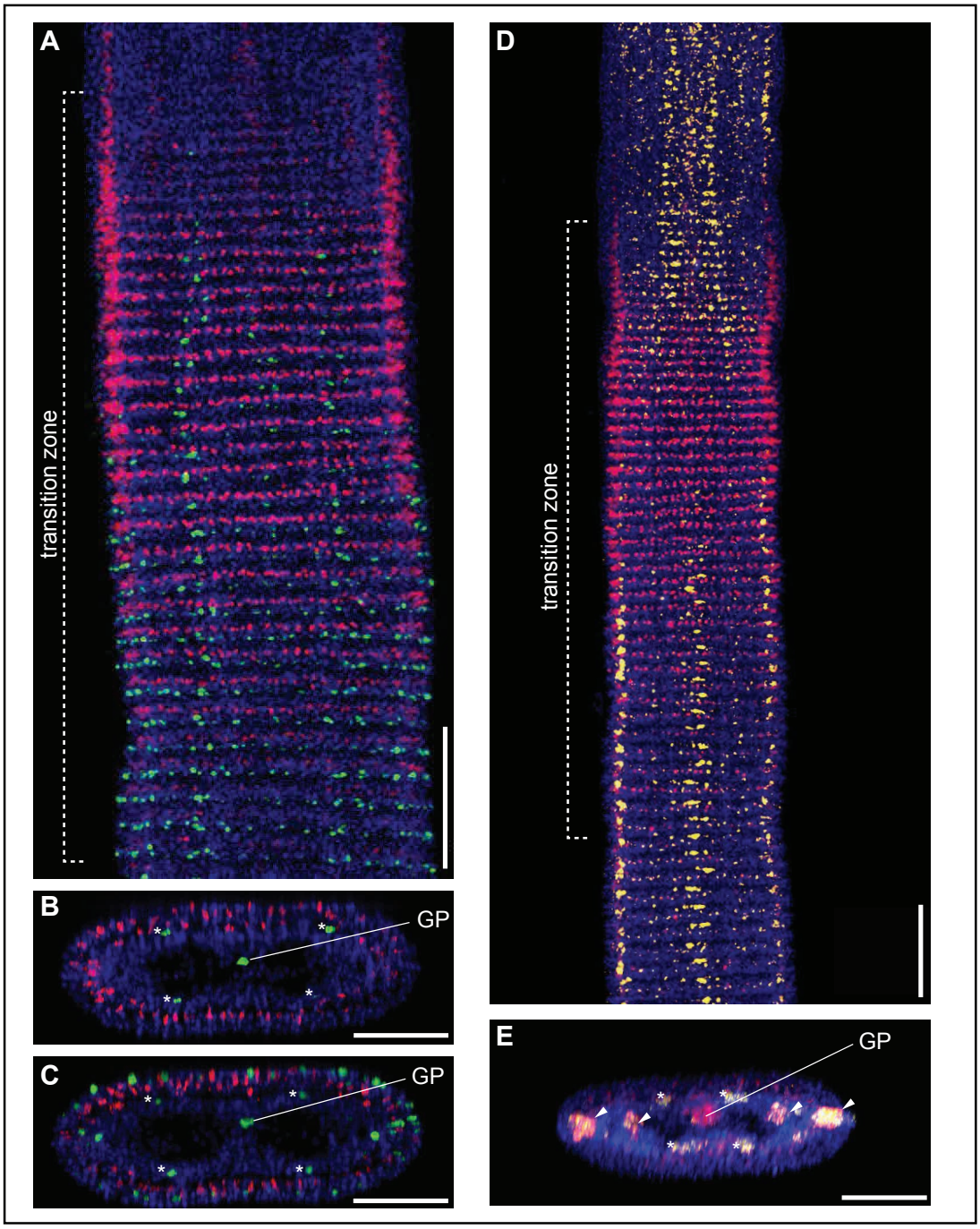

Figure 5.17 Co-expression of *Hmic-Wnt11a* with *Hmic-Wnt1* and *Hmic-Sfl* in adult *Hymenolepis microstoma*. Co-expression of *Hmic-Wnt1* and *Hmic-Wnt11a* in A) the transition zone B) cross section of the early transition zone and C) cross section of the late transition zone. Four nodes of *Hmic-Wnt1* expression are observed where the median nerve cords run the length of the body (indicated by asterisks). Co-expression of *Hmic-Wnt11a* and *Hmic-Sfl* in D) the transition zone and E) cross section in the early transition zone. Co-localisation is observed (arrowheads) and can also be seen where the median nerve cords are situated (asterisks). GP = genital primordia. *Hmic-Wnt1* signal is shown in green, *Hmic-Wnt11a* in pink, *Hmic-Sfl* in yellow and DAPI in blue. Bars: 100 μm

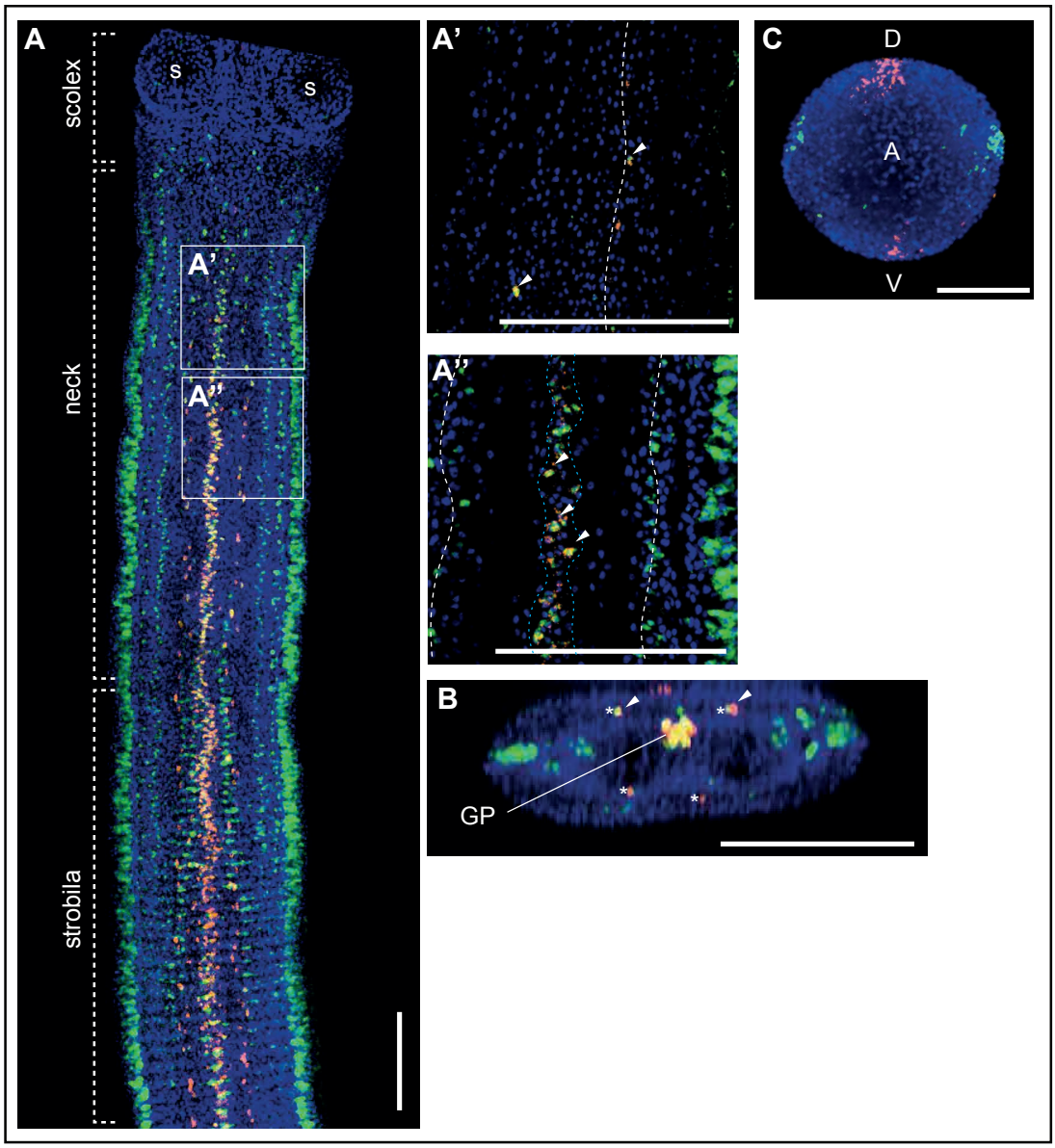

Figure 5.18 Co-expression of *Hmic-Sfl* with *Hmic-Hh* in adult and larval *Hymenolepis microstoma*. Co-expression of *Hmic-Sfl* and *Hmic-Hh* in A) the adult worm. A') In the early neck, co-expression occurs (arrowheads) and is observed along the position of the median nerve cords (dashed line). A'') In the mid-neck, co-localisation (arrowheads) is observed more in the central stripe that represents the genital primordia (bound by dashed blue lines) whilst less co-localisation is observed along the median nerve cords (dashed white lines). B) A cross section through the neck shows co-localisation in the genital primordia. The positions of the median nerve cords are indicated by asterisks, and co-localisation surrounding these nerves is sometimes observed (arrowheads). C) Anterior view of stage I larvae. *Hmic-Hh* expression is observed along the dorsoventral axis whilst *Hmic-Sfl* occurs along the anteroposterior axis. A = anterior pole, D = dorsal pole, GP = genital primordia, s = suckers, V = ventral pole. *Hmic-Sfl* signal is shown in

green, *Hmic-Hh* in pink and DAPI in blue. Adult bars: 100 μm, larval bars: 50 μm

Double staining of these two factors in early larvae confirm this, as they do not co-localise (Fig. 5.18 C). The resulting images confirm that *Hmic-Sfl* is expressed in lateral stripes along the border of the DV axes, whilst the expression of *Hmic-Hh* runs perpendicular to *Hmic-Sfl* through the mid-line in medial stripes along the DV axis.

In adult worms, however, co-localisation is apparent in the neck, before the onset of strobilation (Fig. 5.18 A-B). Here, dFISH shows multiple cells that express both *Hmic-Sfl* and *Hmic-Hh*. These are in the central stripe of the genital primordia and the expression of both factors in association with the nervous system, around the median nerves. Co-expression is not observed in all cells, however with less co-localisation observed in cells associated with the median nerves. After the onset of strobilation, no co-expression of *Hmic-Sfl* and *Hmic-Hh* is observed.

5.3 Discussion

5.3.1 Wnt signalling patterns the AP axis of larval planarians and confirms the scolex as anterior

Throughout the Bilateria, canonical Wnt signalling has a conserved role in the specification of the AP axis and the expression patterns of many Wnt factors are conserved (Petersen and Reddien, 2009; Niehrs, 2010). *Wnts* involved in canonical signalling are expressed towards the posterior whilst antagonists (e.g. *Sfrp* and *Sfl*) are found towards the anterior pole. This is

also true of free-living planarians during regeneration and homeostasis tissue replacement. In these flatworms, Wnt/β-catenin dependent signalling is required for specification and maintenance of the planarian AP axis (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008; Gurley et al., 2010; Almuedo-Castillo et al., 2012). Comparing the expression patterns in *H. microstoma* larvae with those observed in planarians highlights some remarkable similarities between the two flatworms. Orthologs of the 'posterior' *Wnt* genes of planarians (i.e *Wnt1*, *Wnt11a* and *Wnt11b*) are expressed towards the hook-bearing pole of early stage *H. microstoma* larvae whilst the secreted antagonists *Sfrp* and *Sfl* are turned on in the opposite pole. Likewise, the posterior expression of *Fz4* observed in planarians (Gurley et al., 2008) is conserved in *H. microstoma* larvae. Furthermore, the anterior and lateral expression patterns of the ligands *Wnt2* and *Wnt5* (that are not involved in AP axis formation) are also conserved between *H. microstoma* and planarians. The congruency between the early development of *H. microstoma* larvae and planarians strongly indicates the conservation of canonical Wnt signalling to specify the AP axis in tapeworms (Fig. 5.19). Different flatworm groups employ various developmental patterns during early development (Martín-Durán and Egger, 2012) that make comparisons between groups difficult, when based on morphology alone. Expression patterns of *Wnts*, their co-receptors and antagonists during early larval development bear striking similarities to the later stages of planarian embryogenesis (Martín-Durán et al., 2010) and established patterns in adults (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008; Adell et al., 2009; Yazawa et al., 2009; Gurley et al., 2010; Almuedo-Castillo et al.,

2011; Reddien, 2011; Almuedo-Castillo et al., 2012). The early stages of therefore be considered to represent the phylotypic (Slack et al., 1993; larval *H. microstoma* development, after the onset of metamorphosis, can Abzhanov, 2013) (i.e. conserved developmental) stage of cestode development.

The polarised expression patterns of these Wnt factors in larvae finally help to answer the unresolved question of tapeworm AP polarity (Koziol et al. 2016). For decades, debates have argued over the 'true' anterior and posterior of the tapeworm (reviewed in Chapter 1 and Stunkard, 1962; Minelli, 2003; Olson, 2008). Expression of posterior *Wnts* in the so-called 'functional anterior' (i.e. the pole bearing the hooks) shows this to be the posterior pole of the larval worm. The anterior markers, *Hmic-Sfrp* and *Hmic-Sfl*, are expressed in the opposing pole and remain active throughout oncospheral development as this pole develops into the scolex. Conventionally taken as the anterior, the expression of these molecular markers confirms the tapeworm scolex as anterior (Koziol et al., 2016). This also demonstrates that no reversal of the AP poles occurs between development of the oncosphere and adult worm. Later larval development is more derived, but the posterior identity of the metacestode cyst is confirmed by the expression of posterior Wnt factors (i.e. *Hmic-Wnt1*, *Hmic-Wnt11a*, *Hmic-Wnt11b* and *Hmic-Fz4*) within the cyst tissue and anterior markers within the scolex of the encysted juvenile worm.

Confirmation of the 'true' anterior and posterior of cestodes through molecular means is an important finding within cestodology and more

broadly within the field of evo-devo. This was achieved by comparing parasites within the wider context of their natural affinities, rather than solely with other parasitic animals. The importance of this confirmation was emphasised further by two recent articles highlighting the work by Koziol et al. (2016) (Egger, 2016; Minelli, 2016).

5.3.2 Comparison with development of the *Echinococcus multilocularis* metacestode

A parallel study to the results of larval expression in *H. microstoma* presented here was performed in the larvae of the fox tapeworm, *Echinococcus multilocularis* by Uriel Koziol. This too found conserved expression of anterior and posterior markers in the protoscolex and cyst tissue of the developing metacestode (Koziol et al., 2016). Expression analysis of early development of the germinal layer of the metacestode showed that this tissue is initially posteriorised (through expression of *Wnt1*, *Wnt11a*, *Wnt11b* and *Fz4/B*) and that anterior markers (*Sfrp* and *Sfl*) turn to mark sites before budding of the metacestode tissue. The expression patterns of these AP markers in the *E. multilocularis* metacestode shows strong conservation with the expression patterns of planarians and the developing larva of *H. microstoma*. Despite major differences between *H. microstoma* and *E. multilocularis*, such as asexual development, these conserved expression patterns further the idea that larval development in tapeworms represents the phylotypic stage of cestode development within flatworms.

In planarians, PCGs (such as *Wnts* and *Sfrps*) are produced by muscle cells and provide positional information to neoblasts during regeneration and homeostasis (Witchley et al., 2013). Tapeworms, like all other flatworms studied so far, have a similar population of stem cells (germinative cells), which are the only cells that proliferate (Koziol et al., 2014). Co-staining of Wnt factors and EdU showed there to be no significant expression of PCGs in the germinative cells of *E. multilocularis*, as is observed in planarians (Koziol et al., 2016). Double labelling of *Tropomyosin* (a muscle marker) with either *Wnt1* or *Wnt11a* showed that muscle cells also express PCGs in *E. multilocularis* (Koziol et al., 2016). Given other similarities, such as the conservation of *wnt* expression, we can also expect the muscle cells to be the source of PCGs in *H. microstoma*. Early investigations into this through FISH of *collagen* (a muscle marker) shows that collagen expression is punctate and observed (in cross section) in a ring beneath the tegument (Fig. 5.30, OlsonLab, unpublished). The expression pattern of *Collagen* in adults is extremely similar to the expression of Wnt factors (including *Hmic-Sfrp*, *Hmic-Wnt5* and *Hmic-Fz*s), indicating that muscle cells are likely to be the source of *H. microstoma* PCGs. However, further confirmation through more double labelling is still required.

5.3.3 Juvenile *Wnt* expression and elongation of the primary body axis

Once in the mouse stomach, the juvenile worm is activated and sloughs off its protective cyst wall (de Rycke and van Grembergen, 1966). The newly excysted worm clearly has a defined posterior, by way of discrete expression

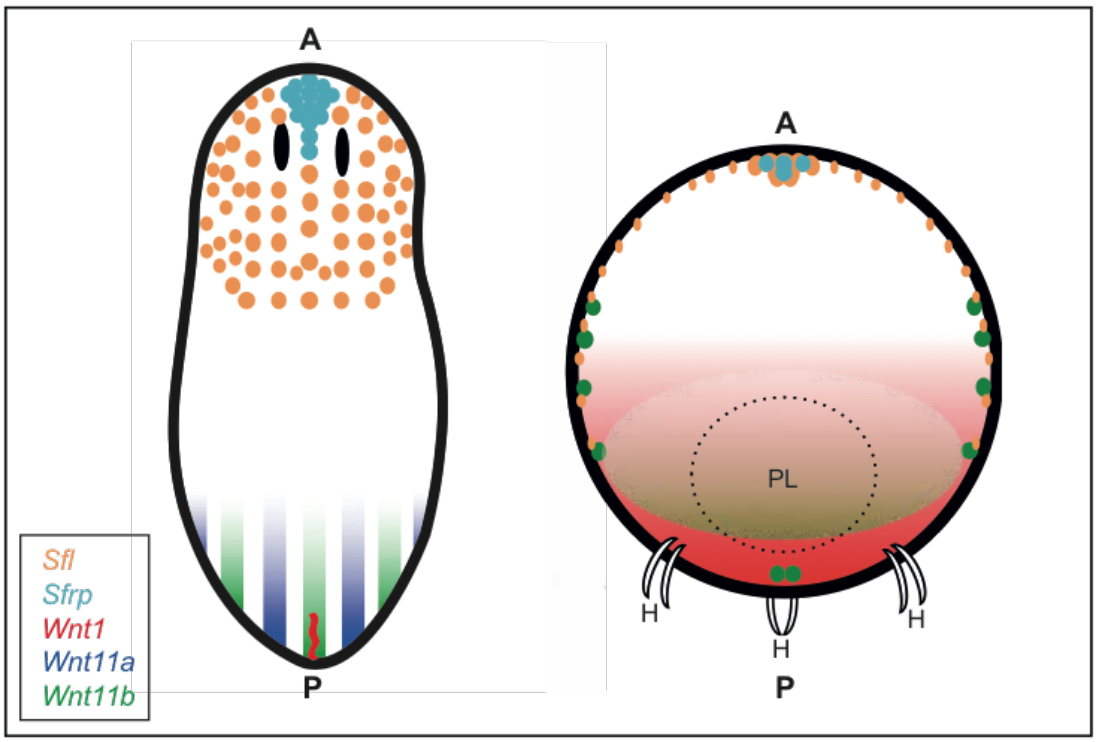

Figure 5.19 Comparison of gene expression patterns in the planarian *Schmidtea mediterannea* with *Hymenolepis microstoma* larva. Expression patters are broadly conserved between these two flatworms and indicates larval tapeworm development to represent the phylotypic stage. A = anterior pole, H = hooks, P = posterior pole, PL = primary lacuna.

of both *Hmic-Wnt1* and *Hmic-Wnt11a* in the pole opposing the scolex 24 hrs after infection in mice. Expression of *Hmic-Wnt1* is restricted to the brood capsule of *E. multilocularis* protoscoleces and is only expressed in a few cells within the posterior of the protoscolex after activation. By comparison, *E. multilocularis Hmic-Wnt11a* is expressed towards the posterior of the developing protoscolex, becoming more restricted after activation in a similar pattern as *E. multilocularis Hmic-Wnt1*. No expression of either of these two genes is visible within the posterior of the juvenile worm of the encysted *H. microstoma* cysticercus, instead expression is within the cyst tissue. As such, it is likely that the expression of *Hmic-Wnt1* and *Hmic-Wnt11a* after excystment of the juvenile *H. microstoma* within the host stomach is similar to *E. multilocularis Hmic-Wnt1* expression, turning on once the juvenile worm is activated.

As the juvenile worm elongates, discrete expression of these two genes within the posterior-most tip continues. By 96 hrs p.i. in mice, worms have begun to strobilate, as observed in previous descriptions (de Rycke and van Grembergen, 1966; Cunningham and Olson, 2010). *Hmic-Wnt11a* expression now expands into stripes that mirror observations of adult expression. As the worms continue to grow, and more segments are formed the 'ladder' of *Hmic-Wnt11a* expression also grows. Unfortunately, expression of anterior markers in juveniles was not achieved, despite multiple attempts, most likely due to degradation of the specimens. In the activated *E. multilocularis* protoscolex, both *Sfrp* and *Sfl* are expressed within the rostellum (Koziol et al., 2016). *Hmic-Sfrp* and *Hmic-Sfl* are expressed

within the scolex of cysticercoids and the scolex and neck of adult *H. microstoma*. Given the expression of these anterior markers in both *H. microstoma* and *E. multilocularis*, it is likely that their expression in pre-strobilar worms will be within the scolex, extending down the neck halting where posterior genes are active. In this way *Hmic-Sfrp* and *Hmic-Sfl* expression are likely to unite the scolex and neck prevent posteriorisation.

5.3.4 Wnt factors are likely to be expressed by muscle cells and are also associated with the nervous system

Several of the factors investigated here (including *Hmic-Fz*s, *Hmic-Sfrp*, *Hmic-Sfl*, *Hmic-Wnt1*, *Hmic-Wnt4*, *Hmic-Wnt5* and *Hmic-Wnt11a*) are expressed in a cylinder of cells within the cortex. In adult *H. microstoma* (and, indeed all tapeworms) there is a tightly associated ring of nervous tissue, germinative cells and longitudinal muscles (Halton and Maule, 2004; Koziol et al., 2010; Rozario and Newmark, 2015) that putatively acts as a 'developmental signalling cylinder' (DSC). This is surrounded by the cortex, including longitudinal and orthogonal muscle layers (Halton and Maule, 2004; Rozario and Newmark, 2015). The muscles of flatworms are unusual, with non-contractile myocytons (containing the nuclei) offset from the contractile myofibril (Halton and Maule, 2004; Witchley et al., 2013). The cortex is thought to be largely filled with these muscle cytons, rather than parenchymal tissue. The punctate expression of Wnt factors in the cortex is consistent with expression around the nuclei of muscle cells rather than the contractile parts of the cells, as seen in planarians (Witchley et al., 2013). As

described previously, several Wnt factors (including *Wnt1*, *Wnt4*, *Wnt5*, *Wnt11a*, *Sfrp-1*, *Sfrp-2* and *Fz4*) are so-called PCGs, that are expressed by muscle cells and provide positional information to migrating neoblasts during regeneration and tissue turnover (Witchley et al., 2013). The muscle cells of *E. multilocularis* express *Wnt* ligands (Koziol et al., 2016) and it is likely that tapeworm muscle cells also express other Wnt factors and PCGs as planarians do. However further confirmation is required from other Wnt factors and muscle markers, such as *Troponin* or *Collagen*.

An example of the positional information PCGs can provide is observed in the expression of *Wnt5* in planarians. It is expressed laterally from the outer border of the CNS along to the ventral DV boundary (Marsal et al., 2003; Adell et al., 2009; Gurley et al., 2010; Almuedo-Castillo et al., 2011). Acting with *Slit* (that is expressed in the midline) (Cebrià et al., 2007; Gurley et al., 2010), *Wnt5* regulates the boundaries of the nervous system by providing a repulsive signal for axon guidance (Almuedo-Castillo et al., 2011). The lateral expression of *Hmic-Wnt5* in larvae and a ring of expression below the tegument in adult *H. microstoma* suggests a similar pattern to planarian *Wnt5* expression. *Wnt5* in tapeworms may also be involved in designating the 'edge' of the worm (through expression in 'lateral myocytons') and regulating the expansion of neuronal growth. It would be interesting to examine the expression of *Slit* in *H. microstoma* to investigate if expression lies 'inside' of the tapeworm nervous system.

The association of *Hmic-Wnt1* and synapsin (and the localisation of *Hmic-Sfl*) along the median nerves suggest that both *Hmic-Wnt1* and *Hmic-Sfl* are expressed within the DSC. In this region, it is difficult to distinguish between different cell types and therefore which cells are expressing Wnt factors. As the muscle cells of other flatworms express *Wnt1* and *Sfl* (Witchley et al., 2013; Koziol et al., 2016), it can be presumed that muscle cells within the DSC are expressing these Wnt factors in *H. microstoma*. The close spatial association between muscles and nerves in the DSC therefore explains the link with *Hmic-Wnt1* and synapsin. It is likely that the muscle cells of *H. microstoma* are providing positional information to germinative cells during tissue turnover and the processes of proglottisation and strobilation in a similar way to that during regeneration and homeostasis in planarians.

5.3.5 Wnt signalling and the development of secondary AP axes during strobilar growth

The co-expression of *Hmic-Wnt1* and *Hmic-Sfl* in the late neck is particularly interesting given that during larval development and post-strobilation these genes are clearly polarised. However, co-localisation of *Wnt1* and *Sfrp* occurs during regeneration of planarians, where both genes are expressed early on, in the anterior blastema (Almuedo-Castillo et al., 2012). It is thought that *Sfrp* inhibits early *Wnt1* activity, resulting in later polarisation of the genes. Co-expression of *Wnt1* and *Sfrp* is also observed during early stages of embryogenesis in *Schmidtea polychroa* before subsequent polarisation in the juvenile planarian that resembles adult expression patterns (Martín-

Durán et al., 2010; Martín-Durán and Romero, 2011). Something similar is surely also in place in the *H. microstoma* neck, where an 'embryonic-like' development of tissue gives rise to nascent segments. Similarly, to where the embryonic tissue or injured adult tissue is reorganising itself in planarians, *H. microstoma* is also reorganising itself as it begins to strobilate. It is most likely that muscle cells are providing positional information to organise and pattern the newly forming segments. The repeated, polarised expression of *Hmic-Wnt1* and *Hmic-Sfl* post-strobilation strongly indicates that these two genes are involved in the patterning of secondary AP axes within segments.

5.3.6 A novel use for *Hmic-Wnt11a* and the definition of a new zone

The activation of *Hmic-Wnt11a* towards the end of the neck and on into early segments is unique when compared to the expression of other genes in *H. microstoma* adults generally. The region *Hmic-Wnt11a* expression spans is often found to be where other genes either turn on or off. In this zone, the expression patterns of *Hmic-Sfl* and *Hmic-Wnt1* are observed to change, transitioning between different patterns that are in place before and after the end of the neck, i.e. pre- and post-strobilation. The expression of *Hmic-Wnt11a* defines a new region of the adult tapeworm that cannot be defined morphologically – which we call the 'transition zone'. Nascent segments are first observed here through the punctate, ladder-like rings of *Hmic-Wnt11a* expression. Once these stripes of expression have been activated and the earliest stages of strobilation are established, co-expression of *Hmic-Sfl* and *Hmic-Wnt1* ceases and polarised stripes of *Hmic-Sfl* and *Hmic-Wnt1* begin.

One explanation to the highly restricted zone of *Hmic-Wnt11a* expression and its perfect correspondence with the first outward evidence of strobilation suggests that *Hmic-Wnt11a* is acting as the effector of segment formation in adult worms and has acquired a novel role in adult development. However, a second explanation could suggest that *Hmic-Wnt11a* expression indicates the early specification of AP polarity within segments. Strobilation in tapeworms occurs through 'pinching', or contraction, of muscle (OlsonLab, unpublished). *Hmic-Wnt11a* is expressed by muscle cells in other flatworms (Witchley et al., 2013; Koziol et al., 2016). As the apparent effector of strobilation, we can presume that *Hmic-Wnt11a* expression in muscle cytons triggers pinching and contraction of muscle fibres and subsequent compartmentalisation of the segments. *Hmic-Sfl* and *Hmic-Wnt1* then provide positional information as to the anterior and posterior of the segment.

The 'intercalary model' of regeneration suggests that cells have positional information and that after injury, intercalation restores missing positional coordinates between juxtaposed tissue (Chandebois, 1979, Chandebois, 1980, Agata et al., 2007). Intercalation directs AP axis regeneration in planarians (Agata et al., 2003) and may also apply to tissue turnover during homeostasis in intact animals (Almuedo-Castillo et al., 2012). One explanation of the expression pattern of *Hmic-Wnt11a* in the transition zone suggests that it triggers the onset of strobilation. The subsequent expression of *Hmic-Sfl* and *Hmic-Wnt1* soon after *Hmic-Wnt11a* turns on, that first co-localise then spread out in the transition zone, suggests intercalation is

continuing the strobilation process by ‘filling in’ between the newly formed axes of segments that are specified by *Hmic-Sfl* and *Hmic-Wnt1*.

Simultaneous suppression of *Wnt1* with each of the three *Wnt11* subclasses in *S. mediterranea* showed that *Wnt11-1* does not cooperate with *Wnt1* in specifying the posterior. Instead, it may be involved in specification and patterning of central regions, as observed by duplication of the pharynx (Sureda-Gómez et al., 2015). Planarian *Wnt11-1* is also thought not to act in a *β-catenin* dependent (i.e. canonical) manner but does cooperate with *β-catenin* dependent Wnt signalling (Sureda-Gómez et al., 2015). This would reflect the integrated cross-talk among Wnt ‘pathways’ proposed by van Amerongen and Nusse (2009). In cnidarians (Guder et al., 2006) and sea urchins (Range et al., 2013) cooperation between *Wnts* belonging to different pathways is essential to pattern the oral-aboral and AP neuroectoderm axes, in the respective groups. However, *Hmic-Wnt11a* is a tapeworm novelty (a paralog with no direct ortholog in planarians (Riddiford and Olson, 2011)) that seems to have switched from a canonical role during larval development to a novel one during strobilar growth. Thus, it seems that both canonical and non-canonical signalling in *H. microstoma* are likely to act together during strobilation in a similar way to the patterning of central regions in planarians.

5.3.7 The adult tapeworm body plan – a type of paratomy?

In most metazoans, AP polarity is continuous along the length of the animal and, in most serially segmented animals the polarity of each segment follows

that of the whole animal. This is also true of tapeworms. Most animals that are segmented add a defined number of new segments from a posterior growth zone so that the newest segments are the most posterior. However, in tapeworms, new proglottides form from an anterior germinative region within the neck and as such, the most posterior proglottides of the strobila are the oldest. In animals that undergo transverse fission, the polarity of the parent and daughter (and all segments) are also continuous. In animals that undergo paratomy, a type of asexual reproduction in which the organism splits along a plane perpendicular to the AP axis, polarity is maintained, and head-to-tail chains of animals or zooids form. Polarity of the AP axis is not always maintained – asexual reproduction by posterior budding in acoels results in a reversal of the AP axis of the new worm in relation to the 'parent' (Sikes et al., 2008). Pre-strobilation, the tapeworm has primary AP polarity, in which the scolex is anterior, and the end of the strobila is posterior (Fig. 5.20). This can be interpreted as the primary AP axis and is formed during metamorphosis. In adult worms, expression of anterior markers defines the scolex and neck as anterior whilst the strobila is posterior. The 'functional' anterior of oncospheres has resulted in the idea that a reversal of polarity occurs in tapeworms (reviewed in Stunkard, 1962). *Hmic-Sfl* and *Hmic-Wnt1* expression in *H. microstoma* confirm that the polarities of segments are not reversed and that the AP axis is preserved from the oncosphere through strobilation. Each segment has its own AP axis that follows that of the entire worm, and can be interpreted as a chain of secondary AP units (Fig. 5.20). Strobilation in tapeworms can be inferred as a form of paratomy in which the

strobila is comprised of a chain of repeating AP units that each have the ability to reproduce.

5.3.8 Interactions between Wnt and Hh signalling

Hh signalling has been shown to act upstream of canonical Wnt signalling in planarians and, along with Wnt signalling, is involved in the establishment and maintenance of polarity (Rink et al., 2009; Yazawa et al., 2009). Given that Hh signalling is responsible for posteriorisation, it is interesting that, in the *H. microstoma* neck, *Hmic-Hh* is co-expressed with an anterior marker, *Hmic-Sfl*. However, *Hmic-Sfl* is also co-expressed with the posterior *Hmic-Wnt1* in the neck. Co-expression between *Hmic-Sfl* and *Hmic-Hh* in a central stripe is likely to be explained by the fact that both Wnt and Hh signalling are involved in patterning and specification of the genital primordia. Fewer cells are found to co-express *Hmic-Sfl* and *Hmic-Hh* surrounding the median nerves and instead, cells expressing either tend to be next to each other. The co-expression of *Hmic-Sfl* and *Hmic-Wnt1* suggests that *Hmic-Wnt1* and *Hmic-Hh* may also, occasionally co-localise. Yazawa et al. (2009) proposed a model in which Hh signals are transported along the planarian central nervous system that then induce Wnt signalling. The expression patterns of *Hmic-Sfl*, *Hmic-Wnt1* and *Hmic-Hh* indicates the strong possibility that the mechanism described by Yazawa et al. (2009) is also in place in tapeworms. In Chapter 4, Hh signalling was shown to be linked with the nervous system. Hh signals transported by neurons could also be directing Wnt signalling

within the neck, prior to strobilation. Loss of function studies (i.e. inhibition of *Hmic-Hh*), however, are required to confirm this.

5.3.9 Wnt signalling and development of the reproductive system

Many of the Wnt factors observed in this study were expressed early in the neck in a centralised stripe, the 'primitive streak' that represents the genital primordia. Later expression shows both canonical and non-canonical Wnt factors are expressed in the developing female reproductive system and the uterus. Combined these patterns strongly suggest a role for Wnt signalling in the patterning, differentiation and development of the female reproductive system in tapeworms, a feature conserved with vertebrates (reviewed in Gerhart, 1999). Wnt signalling is vital for the development of the female reproductive system in mice. Several *Wnt* ligands and *Fz* receptors are required for regulating development of the ovary, uterus and reproductive tract (Miller et al., 1998' Miller and Sassoon, 1998; Parr and McMahon, 1998; Sassoon, 1999; Vainio et al., 1999; Heikkilä et al., 2001; Richards et al., 2002; Ricken et al., 2002; Hsieh et al., 2003), whilst *Sfrp-4* acts as a tumour suppressor in ovarian cancer (Jacob et al., 2012). In fish, however, the requirement for *Hmic-Wnt4* in differentiation of the ovaries is not needed (Nicol et al., 2012). In *D. melanogaster*, Wnt signalling regulates the proliferation and self-renewal of somatic stem cells in the ovary (Song and Xie, 2003; Kirilly et al., 2005). Wnt signalling is clearly involved in the regulation and development of the female reproductive system and the expression patterns of several Wnt factors across the development the

female reproductive system in *H. microstoma* suggests that this role is conserved in tapeworms. Unfortunately, these expression patterns cannot be compared with planarians as most research into planarian development is focussed on the regeneration of asexual strains, rather than sexual development. However, the expression of Wnt factors in the female reproductive system does appear to be conserved amongst *H. microstoma*, mammals and *D. melanogaster*.

Expression in the uterus indicates Wnt signalling is involved in the patterning and development of embryos. It can be assumed that the original AP axis of tapeworms is specified during embryogenesis, as it is in other animals. The expression of Wnt factors during embryogenesis was not explored specifically in this study and is made difficult by this occurring *in utero* in tapeworms. In planarians, *Wnts* and associated factors are expressed during embryogenesis, patterning the early AP axis (Martín-Durán et al., 2010; Martín-Durán and Romero, 2011). Given the expression of these factors in closely related free-living flatworms, they are also most likely expressed during *H. microstoma* embryogenesis, and this likely explains the expression of several *Wnts* in the uterus.

5.4 General conclusions and future directions

The conservation of the Wnt pathway in tapeworms has already been established (Riddiford and Olson, 2011). The results of this chapter indicate that the expression of canonical Wnt factors in *H. microstoma* larvae play a

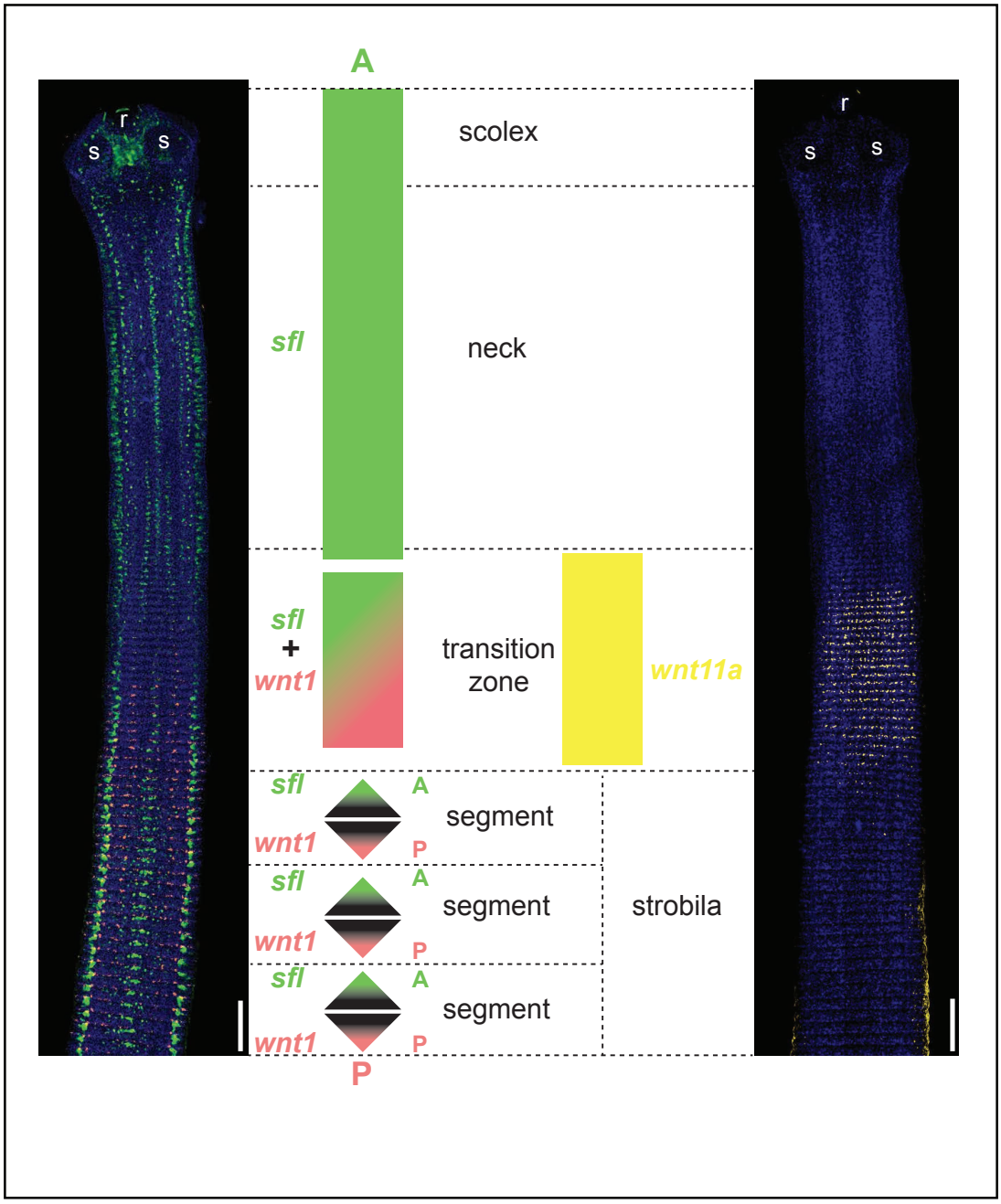

Figure 5.20. Wnt signalling controls AP polarity and strobilation in adult tapeworms. The adult tapeworm has a primary AP axis (in which the scolex and neck are anterior) that is specified earlier during embryonic development. *Hmic-Wnt11a* is a putative effector of strobilation, with expression occurring in the transition zone. Polarised expression of *Hmic-Sfl* and *Hmic-Wnt1* is then observed in every segment, with each having its own secondary AP axis. A = anterior, P = posterior, r = rostellum, s = suckers. *Hmic-Wnt1* signal is shown in green, *Hmic-Sfl* in pink, *Hmic-Wnt11a* in yellow and DAPI in blue. Bars: 100 μm

conserved role in the formation of the primary AP axis in tapeworms and that larvae represent the phylotypic stage of tapeworm development (Koziol et al., 2016). Later development in adults confirms the scolex and neck as anterior and that no reversal of polarity occurs at any point during tapeworm development. Expression of *Hmic-Wnt11a* suggests that it may function as the effector of strobilation whilst *Hmic-Sfl* and *Hmic-Wnt1* are involved in the AP patterning of segments post-strobilation. Although other explanations of *Hmic-Wnt11a* roles are also possible, for example, it may be involved in helping to specify the AP axis of developing segments. Likewise, other genes may act as the effectors of strobilation in *H. microstoma*. Early investigations suggest that *Wnts* and other factors (or PCGs) are likely to be expressed by muscle cells and that they provide positional information to guide germinative cells during proglottisation, strobilation and tissue maintenance. Future work is required to confirm this using known muscle markers such as *Collagen* and other markers used in planarian studies. Expression of some pathway factors in the genital primordia implies a role in proglottisation and later development of the female reproductive system. Gene expression studies of Wnt factors not explored in this chapter (including the remaining *Hmic-Fz*s) will aid in a greater understanding of Wnt signalling in tapeworms. However, the ultimate means with which to fully explore Wnt signalling in *H. microstoma* remains the development of robust functional tools. Once in place, these can be used to knockdown *Hmic-Wnt11a*, to confirm its putative role in tapeworm strobilation.

Chapter 6

Preliminary functional investigations via *in vivo* RNA interference

6.1 Introduction

Important tools with which to investigate gene function are knockdown experiments that work through RNA interference (RNAi) or small molecule inhibition. RNAi allows for gene-specific knockdown of mRNAs (and thus the proteins they encode) by activating endogenous pathways for transcript degradation, whereas small molecule inhibitors block signalling pathways by binding to specific pathway components. When investigating the development of parasitic flatworms, genetic manipulation *in vivo* can be impractical due to their complicated lifestyles. *In vitro* culture systems of entire (or even partial) lifecycles often provide the best settings in which to study parasite development. However, many difficulties persist in culturing helminths *in vitro* and these are the primary reasons why RNAi has yet to become a routine tool for investigating helminths (Hoffmann et al., 2014).

6.1.1 *In vitro* culture of cestodes

Amongst cestodes, the development of adult worms in culture has been achieved in several species e.g. *Moniezia expansa* (Markosi et al., 2003), *Spirometra mansonoides* (Smyth and McManus, 1989), with most work being conducted in the 1960/70s. However, there is no standard cestode culturing procedure and results can be inconsistent, even between different batches of media (Evans, 1980). *In vitro* systems have developed considerably in recent years, especially in species that cause disease in humans. Axenic culturing systems of primary cells of *Echinococcus multilocularis* and its development

from oncosphere to metacestode have been developed (Brehm and Spiliotis, 2008; Spiliotis et al., 2008) as have *in vitro* culture methods of oncosphere to cysticercus development in the pork tapeworm *Taenia solium* (Chile et al., 2016). However, in neither case have contemporary methods for *in vitro* culture of strobilar adults been developed. *In vitro* culture of entire lifecycles has been demonstrated in a handful of species, including *Schistocephalus solidus* and *Hymenolepis* spp (Evans, 1980), but the methods have not been replicated by other researchers.

6.1.2 *In vitro* culture of *Hymenolepis microstoma*

Modifications of techniques developed from the *in vitro* culture of *H. diminuta* and *H. nana* (Berntzen, 1961; Berntzen, 1962) and *S. mansonoides* (Berntzen and Mueller, 1964) lead to the first reported *in vitro* culture of *H. microstoma* (de Rycke and Berntzen, 1967). This was developed further (Seidel, 1971) and the completion of the entire *H. microstoma* system *in vivo* (from egg through to gravid adult) was achieved around 40 years ago (Seidel, 1975; Evans, 1970; Evans, 1980). However, recent attempts to replicate their methods were unsuccessful in achieving the segmentation of adult worms and only resulted in limited growth of juvenile worms (Cunningham and Olson, 2010; Pouchkina-Stantcheva et al., 2013). Juvenile worms removed from the bile duct of infected mice that are transferred to culture media only ‘just’ seem to be maintained in culture. After about 7-10 days, worms begin to degrade, appearing grainy with ‘blebbing’ of the tegument. Around ten days in culture, worms begin to undergo lysis starting

in the posterior of the worm (pers comms, A. Baillie and P. Olson). Despite intensive attempts at developing the culturing methods, it is most likely that differences in media between the original successes and later attempts to be the cause of the failure to induce strobilation and growth (Pouchkina-Stantcheva et al., 2013).

6.1.3 RNAi in flatworms

Perhaps the strongest tool at our disposal with which to investigate gene function is RNA interference (RNAi), which inhibits the gene expression of target transcripts. RNAi is commonplace in planarian developmental investigations (e.g. Petersen and Reddien, 2008; Rink et al., 2009; Yazawa et al., 2009 and Sikes and Newmark, 2013) and is now expanding into parasitic flatworms. Several RNAi methods for various species and life stages of flukes have been produced (e.g. Freitas, 2007, Rinaldi, 2008, Rinaldi, 2009). RNAi methods are also being developed in cestodes (e.g. Mizukami et al., 2010, Spiliotis et al., 2010, Pouchkin-Stantcheva et al., 2013, Hu et al., 2015). RNAi in *H. microstoma* has already been shown to be effective (Pouchkina-Stantcheva et al., 2011) where gene expression was shown quantitatively to be suppressed by up to 80%. Unfortunately, the difficulties in the *in vitro* culture, described above, prevented determination of the developmental outcome.

6.1.4 Pathway interference through chemical inhibition

Broad functionality can also be determined by the use of chemical inhibitors that block signalling pathways. One of these chemicals is 'inhibitor of Wnt production compound 3' (IWP-3), which, as the name suggests is an inhibitor of the Wnt pathway. IWP-3 prevents the secretion of wnts by inactivating porcupine (porc) (Chen et al, 2009); a membrane bound O-acyltransferase that palmitoylates Wnts. Palmitoylation of the Wnt protein occurs in the endoplasmic reticulum and is essential for Wnt signalling (Takada et al., 2006). As such, the inactivation of Porc (and subsequent blocking of palmitoylation) shuts down Wnt activity. IWP-3 treatment of embryo cultures of the American cockroach, *Periplaneta americana*, results in reduced expression of the segmentation marker *caudal*, a reduced growth zone and disrupted segmentation (Chesebro et al., 2012). These authors found that concentrations of IWP-3 between 30 – 40 μM resulted in the strongest phenotypes without inducing death of the embryos. IWP-3 also prevents human embryonic stem cells from producing Wnts (Willems et al., 2011).

A second chemical, DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester) is a specific inhibitor of Notch signaling (Geling et al., 2002). The Notch protein must be cleaved three times before the Notch intracellular domain (NCID) is released and translocates to the nucleus where it then activates transcription of downstream targets of Notch signaling. One of these cleavages, the S3 cleavage, is performed by the γ-secretase complex. DAPT interferes with γ-secretase activity (Dovey et al.,

2001, Sastre et al., 2001) and therefore act to inhibit Notch signaling (Geling et al., 2002). In zebrafish, DAPT treatment impairs somite formation and results in irregularities in somite size, shape and polarity (Geling et al., 2002). DAPT treatment of *P*. *Americana* embryos results in alterations to the expected patterns of the segmental genes *engrailed* and *hairy* (Pueyo et al., 2008) and a reduced expression of *wnt1* coupled with perturbed segmentation (Chesebro et al., 2012). Segmentation of *Helobdella robusta* is disrupted with DAPT treatment (Rivera and Weisblat, 2009) whilst in *Hydra*, inhibition of Notch signalling with DAPT disturbs the maintenance of head morphology (Münder et al., 2013b).

6.1.5 Development of new functional tools within *Hymenolepis microstoma*

There is a need to develop new approaches with which to investigate gene function in *H*. *microstoma*. Due to difficulties in traditional culturing methods, we are unable to successfully grow and maintain *H*. *microstoma in vitro*. Coupled with this, attempts to soak cysticercoids in dsRNA prior to inoculating mice were unsuccessful. Assays using fluorescently-labelled siRNAs showed that the RNA stayed in the cyst tissues of the cysticercoid and failed to penetrate the juvenile worm (Pouchkina-Stantcheva et al., 2013). As such, I have developed a novel approach with which to investigate RNAi and chemical pathway inhibitors. This is an *in vivo* method that effectively ‘soaks’ *H*. *microstoma* larvae *in situ*, in the haemocoel of the intermediate host *Tribolium confusum*. By injecting dsRNA or chemical inhibitors directly into the haemocoel of infected beetles, larvae will be

‘exposed and soaked’, absorbing the treatment before encystment occurs. This approach borrows from injection protocols that are standard practice in RNAi investigations of *Tribolium* spp (Posnien et al., 2009). The results of this chapter (although extremely primary) provide the first steps in the development of a promising method with which to investigate gene function in *H. microstoma*.

6.2 Development of an *in vivo* approach to RNAi for functional analyses

RNA interference approaches targeting adult development are hampered by the limitations of *in vitro* culture (Pouchkina-Stantcheva et al., 2013). New approaches that either improve the success, or remove the need for *in vitro* culture need to be developed. Through an International Training and Fieldwork Award funded by the British Society for Parasitology, I visited the laboratory of Dr Nico Posnien at the University of *Göttingen*, Germany to learn how to synthesise dsRNA and inject *T. confusum*.

6.3 Results

6.3.1 Injection of *Tribolium confusum*

Adult *T. confusum* were starved for one week before being exposed to macerated, gravid *H. microstoma* tissue for 24 hr. Beetles were transferred to a fresh petri dish and were anaesthetised by placing the dish on ice. Once beetles had stopped moving, they were individually placed under a

dissecting microscope using soft forceps. Beetles were gently squeezed until softer tissue towards the posterior of the animal became visible. The beetles were manipulated so that a micropipette (attached to a micromanipulator) could be inserted laterally at an angle of ~60^{o}. Micropipettes were inserted into the posterior or exposed joints (e.g. where the abdomen and thorax meet, or leg joints) (Fig. 6.1). Approximately 2-5 ul dsRNA, IWP-3 or DAPT was injected into the beetles until the pressure in the haemocoel became too great. This was visualised by any combination of the following: the posterior pushing out further and swelling; the head, thorax or abdomen spreading away from each other; leg segments spreading away from the body or other leg segments; no more solution could be injected and began to seep out of the injection site. The pressure in the syringe was then reduced and the micropipette quickly removed to prevent injury to the beetles. Beetles were left to recover and allow the posterior to retract and were fed *ad libitum* on flour (as described above). Beetles were dissected 4-9 days post infection (d.p.i.) and larvae were removed. Larvae were viewed under a dissecting microscope and imaged and sub-sets were stored in RNAlater for subsequent qPCR analysis.

6.3.2 Injection with dsRNA

The *in vivo* approach to RNAi was initially trialled in Germany by injecting *T. confusum* haemocoels with *Hmic-Post-2* dsRNA (Pouchkina-Stantcheva et al., 2013) but was confounded by the beetles being refractory to infections. Beetles were exposed to two independent rounds of injections and did

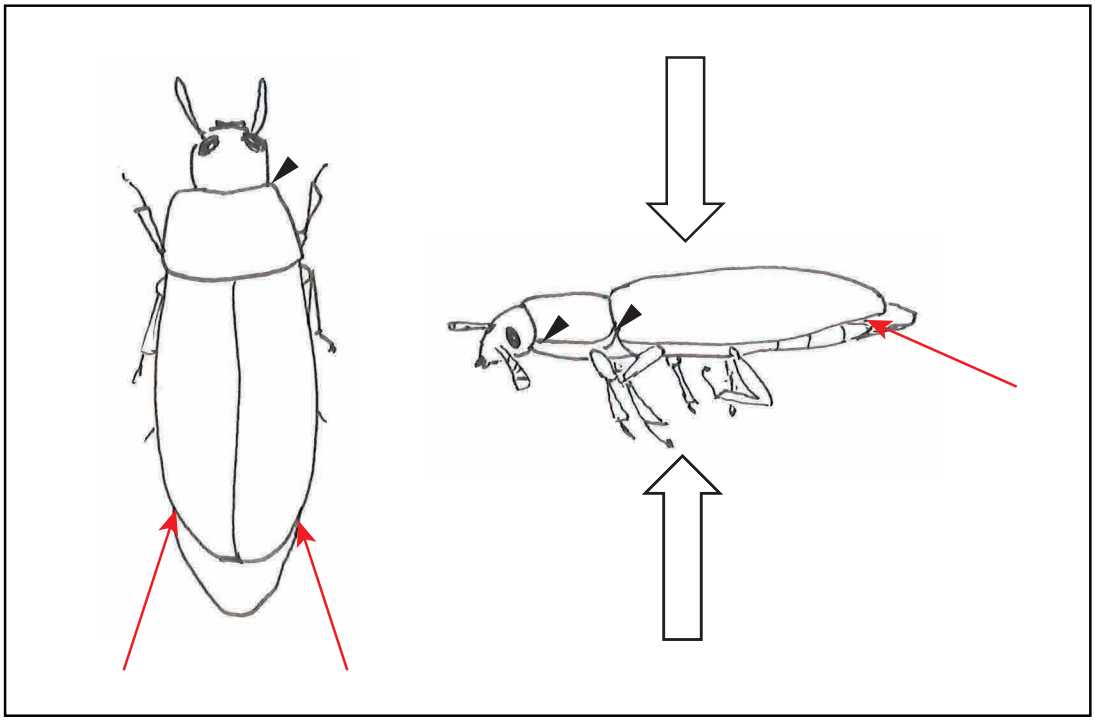

Figure 6.1 The injection of *Tribolium confusum*. After anaesthetising on ice, beetles were injected, either with chemical inhibitors of the Notch and Wnt pathways or *Hmic-Post-2* dsRNA. White arrows show pressure applied to the beetle. Red arrows show where beetles were preferentially injected, whilst arrowheads indicate alternative injection sites.

survive the injection process, but subsequent dissection showed no infection despite exposure to *H. microstoma* eggs. Beetles from the German colony were brought back to the UK where a second attempt to infect them with *H. microstoma* also failed.

6.3.3 Injection with small molecule inhibitors

Once back in the UK, the injection of small molecule inhibitors (rather than RNAi) was focused on as these have known effects on pathway inhibition. Firstly, assays to monitor the survival of injected beetles with water and various concentrations of DMSO (a necessary carrier of the small molecule inhibitors) were performed. These were largely successful with relatively few beetles dying after the injection procedure with only water after one day and the majority still alive after one week. Mortality increased with increasing concentrations of DMSO, with over 50% mortality of beetle injected with 4% DMSO after four days. Injections using 50, 75 or 100 μM/ml DAPT + 1% DMSO did not result in any obvious phenotypic change in the *H. microstoma* larvae recovered (data not shown). *H. microstoma* larvae were stored in RNALater at -20 ^{o}C for future analysis.

Injecting *H. microstoma* larvae with IWP-3 did appear to induce a phenotypic change in recovered larvae. Results were strongest in larvae recovered from beetles injected with 40 μM/ml IWP-3 + 1% DMSO (Fig. 6.2). These larvae showed both delayed development and changes in morphology. *H. microstoma* larvae recovered 4 d.pi. were much smaller than expected

(based on general observation) and the majority had failed to develop past stage I (Fig. 6.2). 7 d.p.i., the developmental differences were much more apparent. Here, larvae were observed that appeared to have stopped developing or were severely delayed in their development as they were very small and spherical in appearance, similar to stage I and II larvae (as staged according to Voge (1964)). Many of the larger spherical larvae had a denser centre and a primary lacuna could not be discerned. Very few larvae had fully developed into metacestodes, unlike the controls, in which the majority had reached either stage V or IV (as staged according to Voge (1964)) (Fig. 6.2). The strongest phenotypic change was observed in larvae that seemed to be between stages III and IV and could be classed into two types. First, those with an extremely narrow constriction of tissue around the point where, during normal development, the anterior tissue of the larva is withdrawn into the posterior and enveloped by the posterior cyst wall. The anterior-most apex also appeared to have started to undergo rostellar development (although stunted), which is not usually observed during collections under normal conditions. Second, those in which the cavity is enlarged, spanning into both hemispheres with some appearing highly symmetrical (Fig. 6.2). However, the primary result appeared to be arrested development of the larvae.

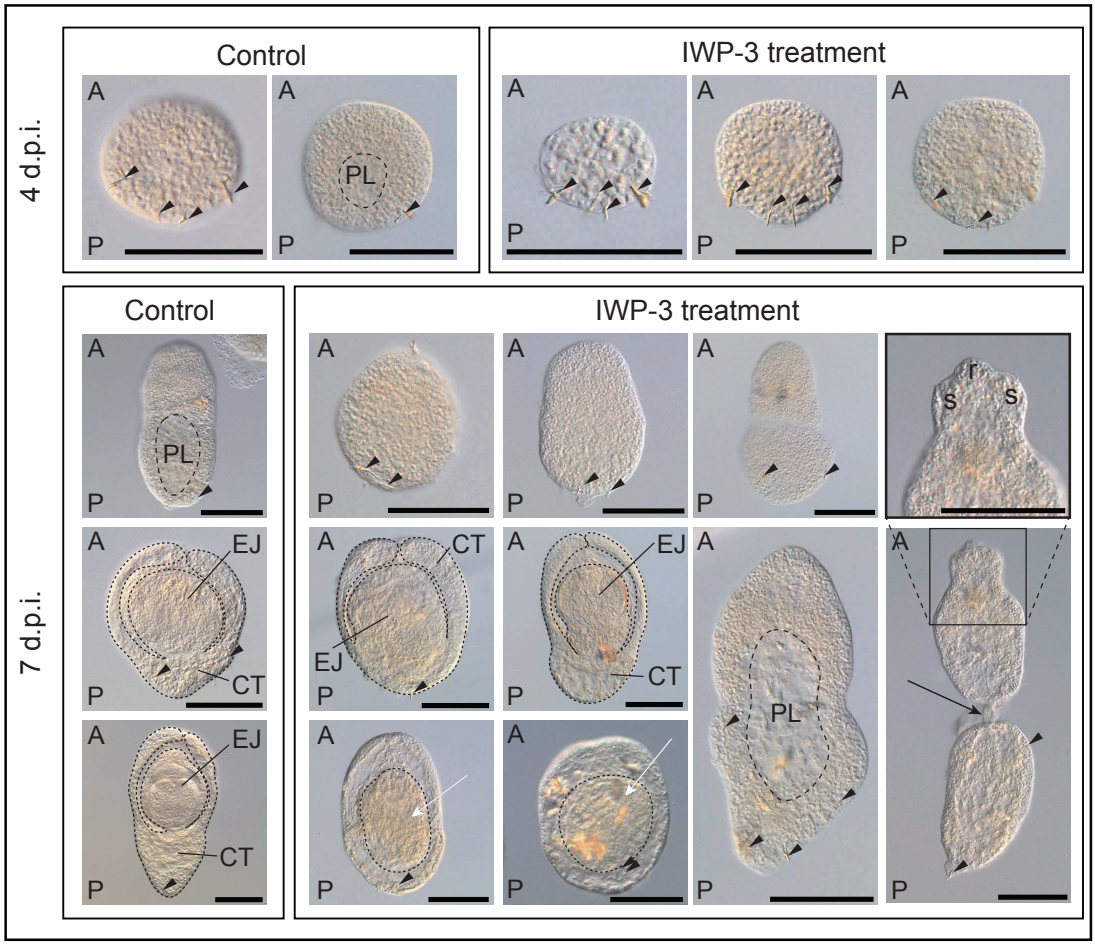

Figure 6.2 Phenotypic changes are observed after injections with IWP-3. *H. microstoma* larvae recovered 4 or 7 days post infection (d.p.i.) from *Tribolium confusum* that were injected with either 2-5 μl 1% DMSO or 2-5 μl 40 μM/ml IWP-3 + 1% DMSO. Larvae recovered 4 d.p.i. after control treatment were either Stage I or II (as according to Voge, 1964). The majority of larvae 4 d.p.i. that were effectively 'soaked' in 40 μM/ml IWP-3 + 1% DMSO were also at Stage I or II, although some smaller oncosphere-like individuals were observed. 7 d.p.i., most larvae collected from the control treatment had reached Stage V, with some Stage IV and even fewer Stage III also observed. The most wide-ranging phenotypes were seen in larvae 7 d.p.i. from beetles injected with IWP-3. These included delayed development with Stages I and II still being recovered. Stage III-IV larvae were also recovered, although based on general observations, there were fewer more developed larvae than in the control treatment. The most extreme phenotype recovered was observed in larvae that seemed to have failed to encyst after reaching Stage III. These larvae had a constriction of tissue (black arrow) and the scolex had started to develop (box). Other phenotypes included Stage III larvae that appeared to have an extremely symmetrical primary lacuna within both hemispheres of the larvae and larvae with an extremely dense centralised region of tissue (white arrows) with no discernible features other than hooks. Arrowheads point to hooks. A = anterior pole, CT = cyst

tissue, EJ = encysted juvenile, P = posterior pole, r = rostellum, s = suckers. Bars: 100 μm

6.4 Discussion

6.4.1 A refractory strain of *Tribolium confusum*

The first rounds of injections were confounded by an inability to infect the beetles with *H. microstoma*. As a second trial using beetles from Germany also proved refractory to infection, it is most likely that these initial experiments failed due to this particular strain of *T. confusum*. As quantitative suppression of a *Hox* gene was shown to be successful via soaking of juveniles *in vitro* without electroporation (Pouchkina-Stantcheva et al., 2013), it is unlikely that dsRNA could not also penetrate developing larvae *in vivo*. Repeating the injection of dsRNA with the UK *T. confusum* strain may lead to successful, gene-specific suppression.

6.4.2 Further analysis is required for confirmation of the technique

In vivo soaking with DAPT did not result in any obvious phenotypic changes. However, further analysis (i.e. WMISH or qPCR) needs to be performed to confirm disruption of the pathway. A factor that would most likely be informative in the determining success of injecting with DAPT would be the downstream targets of Notch signalling, members of the *Hairy/Enhancer of split* (*Hes*) complex. This has been demonstrated in *H. robusta* in which levels of *Hes* were reduced by 30% in individuals treated with DAPT. As IWP-3 seemed to work, it is likely that the Hedgehog pathway may also have been inhibited but did not result in a morphological change. Several

investigations have reported that once DAPT treatment is removed, normal phenotypes resume during growth (Münder et al., 2013a; Pueyo et al., 2008), multiple injections of DAPT may be required to induce a stronger phenotype.

The results from IWP-3 proved more promising. Disruption of Wnt signalling in *H. microstoma* larvae clearly results in developmental abnormalities, as observed in morphology and delayed development (Fig. 6.2). Again though (as with DAPT treatment), confirmation is still needed. This could include qPCR of Wnt targets (such as Axin) or WMISH of the anterior marker *Hmic-Sfrp*. In planarian systems (and indeed other metazoans), disruption of 'posterior' *Wnts* via RNAi results in an anteriorisation of animals (reviewed in Petersen and Reddien, 2009). In mild cases this is a reduction of the tail and in extreme ones, hypercephalisation. *Wnt1* loss-of-function in planarians leads to 'tailess' phenotypes and in more extreme cases, double-headed individuals (Adell et al., 2009). Some of the *H. microstoma* larvae recovered 7 d.p.i. after injection of 40 μM/ml had a large, centralised cavity and were highly symmetrical (Fig. 6.2B). In a minority of these specimens, it appeared that the *H. microstoma* larvae had a reduced posterior. As IWP-3 acts to prevent the secretion of Wnts, it would be predicted that the establishment of the *H. microstoma* larval posterior pole is disturbed, resulting an anteriorised phenotype and possibly, even a second anterior pole (Fig. 6.3).

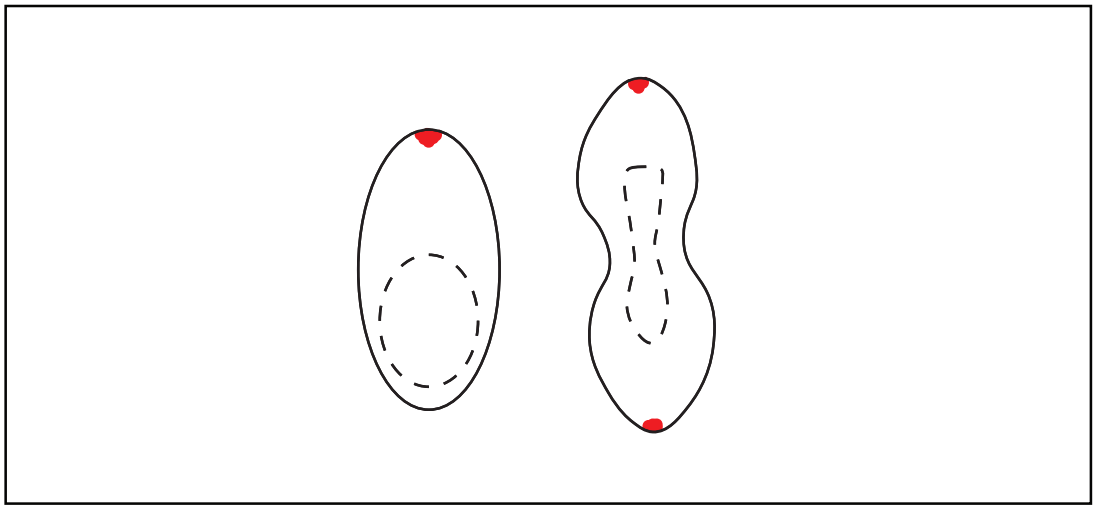

Figure 6.3 Predicted expression of *Hmic-Sfrp* in larval *Hymenolepis microstoma* after treatment with IWP-3. During normal development of *H. microstoma* larvae (left), the anterior pole is defined by expression of the anterior marker *Hmic-Sfrp* (red). In larvae whose development is disrupted by IWP-3 treatment (right), it is predicted that the secretion of *Hmic-Wnt*s will be disturbed resulting in anteriorised larvae. One possible predicted phenotype includes larvae that possess multiple anterior poles, as observed after knock-down of *Wnt1* in some planarians (Adell et al., 2009). This could be tested by investigating the expression of *Hmic-Sfrp*, resulting in the observation of two clusters of expression (red).

6.4.3 Limitations of the technique

The total volume of solution that can be injected into each beetle varies from individual to individual. And whilst the concentration of the solution remains the same whether using 2 or 5 μl, the volume of haemolymph within the haemocoel cannot be controlled. Therefore, the absolute final concentration of dsRNA or chemical inhibitor that larvae are exposed to will vary, as the IWP-3 will be diluted to a small amount by the haemolymph. This is something that cannot be controlled. However, studies investigating early *T. confusum* development that this method is based on, face similar difficulties

(e.g. Posnien et al., 2011). Data resulting from these studies can still be interpreted in a meaningful way. I see no reason why any future experiments investigating gene functionality in *H. microstoma* using the methodologies described here could not also produce significant data relating to tapeworm larval development.

Whilst RNAi acts in a gene specific manner, small molecule inhibitors are universal and may therefore affect the host as well as the parasite. However, as the Wnt and Notch pathways are primarily involved in embryonic development, it was reasoned that they would show little effect on the adult beetles. This appeared to be the case, but future work using RNAi against parasite-specific targets will avoid any potential confounding effects of small molecule inhibitors when employing an *in vivo* approach.

Preliminary results presented here provide the first steps towards developing an effective in vivo approach to RNAi in *H. microstoma*. Whilst this method may prove effective for investigating larval development, it could also be carried over and be used to investigate adult development by inoculating mice with manipulated larvae. This would alleviate the need for in vitro culture of adult worms.

Chapter 7

General discussion

7.1 Introduction

The results presented in this thesis provide the first insights into the expression patterns of three developmental signalling pathways in the tapeworm *Hymenolepis microstoma* and will help in our understanding of tapeworm development. The primary aims of this thesis were to investigate the potential role of the Hedgehog (Hh), Wnt and Notch signalling pathways in specifying the anteroposterior (AP) axis of *H. microstoma* and their function (if any) during strobilation. The expression patterns of Wnt pathway factors suggests that Wnt signalling is involved in proglottisation, strobilation and patterning of the anteroposterior (AP) axis of *H. microstoma*. Hh signalling may play a role in the specification of the posterior during larval development, but is more clearly linked with the nervous system and proglottisation in adult worms. Notch signalling may play a role in proglottisation and is also potentially involved in strobilation and the specification of AP axes in segments. All three pathways were found to be active throughout development and were not exclusive to one particular event.

7.2 Moving away from labelling genes

The three pathways were selected, in part, due the description of factors within these pathways (i.e. *Hh*, *Notch* and *Wnt1*) being described as so-called ‘segmentation’ or ‘segment polarity genes’. Strobilation arose *de novo* in eucestodes (Olson et al., 2001; Olson and Tkach, 2005) and is likely not

homologous with segmentation observed in other animals (Seaver, 2003). Despite this, the serial repetition of units is conserved between strobilation and 'true' segmentation and it was hypothesised that the three pathways may have been co-opted to control strobilation.

Many of the descriptions of these signalling systems (Notch, Hh and Wnt) are based on early investigations stemming from *Drosophila melanogaster*. It is widely accepted that *D. melanogaster* development is highly derived, despite this, comparisons are often made back with this model system and literature often still refers to 'segment polarity genes' (e.g. Baker, 1987; Shigeki et al., 1993; Kadowaki et al., 1996; Simonnet et al., 2004; Dray et al., 2010). As research is broadening out into new systems and models, it is becoming increasingly apparent that a universal role of these genes as 'segmentation' genes is not conserved (Seaver and Kaneshige, 2006; Franke et al., 2014; Janssen and Budd, 2016), especially when considering their roles in animals that are not segmented (Vellutini and Hejnol, 2016)!

7.3 Answering questions relating to anteroposterior polarity

Debate amongst cestodologists have centred around asking what part of the worm is anterior (Stunkard, 1962; Minelli, 2003; Olson, 2008). Through a molecular comparative developmental approach examining the expression patterns of conserved Wnt signalling factors that control AP polarity across the Metazoa (Petersen and Reddien, 2009; Niehrs, 2010), this has finally been resolved (Koziol et al., 2016). In *H. microstoma* larvae, the pole

opposing that bearing the hooks goes on to develop into the scolex. This pole is anterior and is identified as such, by expression of the anterior markers *Hmic-Sfrp* and *Hmic-Sfl*. Conversely, posterior *Hmic-Wnts* are expressed towards the hook bearing pole and within cyst tissue. The expression of posterior markers in the so-called 'functional anterior' of larvae confirms that the hook-bearing pole is posterior, with no reversal of polarity.

The expression patterns of Wnt factors in *H. microstoma* larvae strongly resemble those observed in planarians (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008; Adell et al., 2009; Yazawa et al., 2009; Gurley et al., 2010; Martín-Durán et al., 2010; Almuedo-Castillo et al., 2012). As such, the larval development of tapeworms can be postulated as the 'phylotypic' stage, showing conservation with free-living flatworms (Fig. 5.19) (Koziol et al., 2016). In planarians, the default state of tissues appears to be 'posterior' with anteriorisation requiring the repression of *β-catenin* (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). The expression patterns observed in *H. microstoma* larvae imply that this feature is conserved between tapeworms and planarians. These expression patterns confirm that no reversal of polarity occurs between larval development and the adult and also, that the scolex is anterior (Koziol et al., 2016 and Chapter 5). Although, conventionally, this is generally presumed to be the case, this was an important finding within the fields of cestodology and evo-devo (Egger, 2016; Minelli, 2016).

Expanding this research to investigate Wnt signalling in adults shows that the scolex, together with the neck, represents the anterior pole of the adult worm. This can be seen via the expression of *Hmic-Sfrp* that is restricted to the scolex and neck and *Hmic-Sfl* that has a distinct expression in the neck that is altered post-neck, in the strobila. The expression of *Hmic-Wnt1* and *Hmic-Sfl* highlights that the polarity of individual segments mirror the AP orientation of the adult worm and larvae. Once again, this confirms that no reversal of polarity occurs at any point of the *H. microstoma* lifecycle. Finally, the employment of Wnt signalling to pattern the AP axis throughout metazoans (Petersen and Reddien, 2009; Niehrs, 2010) is conserved amongst tapeworms.

7.4 *Hmic-Wnt11a*, the transition zone and strobilation

The body of the adult tapeworm is traditionally divided into three regions: the neck, scolex and strobila (Fig. 1.3). The expression of the posterior *Hmic-Wnt11a* defines a new region that has not previously been suggested on the basis of morphology, which we refer to as the transition zone (Fig 5.20). This region is located towards the end of the neck and into the start of the strobila, before segments become morphologically visible. The transition zone has only been identified through a developmental genetic approach and is the source of new segments. Gene expression within the strobila of *H. microstoma* is often found to be restricted to either pre- or post-neck, or dramatically alter its pattern after the neck. *Hmic-Wnt11a* is the only gene so far, whose expression is activated across this border, and its expression

defines the boundaries of the transition zone. Whilst *Hmic-Wnt11a* is a candidate effector of strobilation, it would certainly be interesting to investigate if any other genes are up-regulated exclusively in the transition zone. Transcriptomic profiling of the transition zone would allow for a targeted screening approach with which to investigate other factors within this region.

7.5 The potential role of *Wnt11a* in other tapeworms

There are some tapeworm species that display unusual forms of strobilation. For example: caryophyllideans are neither proglottisised or strobilate (Fig. 1.6); spathebothriideans are proglottisised but are not strobilate (Fig. 1.6); and, in the case of *Anantrum tortum*, a secondary loss of strobilation. Finally, there is the highly unusual delay of proglottisation that is observed in *Haplobothrium globuliforme*. In this species, juveniles develop into a primary worm that does not develop sexually. The strobila of the primary worm develops pseudoscoleces, behind which a secondary worm grows and develops sexually. These secondary worms eventually bud off and reattach themselves further down the host gut. It would be highly interesting to investigate the developmental expression patterns of *Wnt11a* in species with these uncommon forms of strobilation. Unfortunately, the scope of this project prevented the exploration of *Wnt11a* expression in other tapeworms. However, this should be examined in the future, as it is likely to result in fascinating comparisons.

Based on the expression of *Hmic-Wnt11a* across the development of *H. microstoma*, some predictions of *Wnt11a* expression can be made. Firstly, in caryophyllideans (such as *Atractolytocestus huronensis* which is neither strobilate nor proglottisised), *Wnt11a* is likely to be expressed in the posterior of the worm, with expression possibly mirroring either the discrete foci observed in Stage V *H. microstoma* larvae (Fig. 5.2 D) and activated *E. multilocularis* protoscolex (Koziol et al., 2016) or the posterior expression of *Hmic-Wnt11a* in pre-strobilar *H. microstoma* (Fig. 5.12). Secondly, in *H. globuliforme* (that displays an unusual form of paratomy in which the only secondary strobila develops proglottides), *Wnt11a* expression likely to be activated behind the pseudoscoleces as the strobila of the secondary worms develop. As a side note, it would also be interesting to investigate the anterior markers *Sfrp* and *Sfl* in primary *H. globuliforme*. Are anterior markers expressed in the pseudoscoleces that develop along the strobila of the primary worm? Are they expressed in a similar manner between the primary and secondary scolex? These are interesting developmental questions that should be probed further in the future.

7.6 The 'developmental signalling cylinder'

The nervous system is linked with regeneration in planarians. Knockdown through RNAi of factors expressed by neurons leads to a reduction of neoblast proliferation (Reddien et al., 2005) and inhibits regeneration (Brockes and Kumar, 2008). In *Dugesia japonica*, expression of the neoblast proliferation marker *MCM2* adjacent to the ventral nerve cords (VNCs)

suggests that proliferation of neoblasts can be induced by neuronal cells (Salvetti et al., 2000). Planarian neoblasts certainly seem to be controlled by signals secreted by the nervous system, a role that is conserved with vertebrates (Rossi et al., 2012). The planarian CNS is dotted with neurosecretory cells and it is thought that substances are secreted close to target cells due to a lack of a circulatory system (Cebrià, 2007). It has been suggested that planarian Hh signals are transported along nerve cords, inducing downstream factors such as *Wnts* (Yazawa et al., 2009). Recently, neurons were found to signal to adjacent glia and neoblasts that pattern and maintain planarian neurogenesis during homeostasis and regeneration (Currie et al., 2016; Wang et al., 2016).

Position control genes (PCGs) are regionally expressed genes (that include several Wnt factors) whose inhibition results in abnormal patterning (Reddien, 2011). In planarians, the source of these PCGs are muscle cells (Witchley et al., 2013) and the expression profile of PCGs within these muscle cells are extremely plastic, with the ability to change in response to injury (Witchley et al., 2013; Reuter et al., 2015). Together this suggests a model in which muscle cells provide positional information to neoblasts during regeneration and homeostasis (Witchley et al., 2013). Furthermore, in another link to neurogenesis, the PCG *Wnt5* acts together with *Slit* to restrict the growth of neurons and regulate the borders of the CNS in planarians (Almuedo-Castillo et al., 2011).

As in planarians, muscle cells are also the source of *Wnts* in tapeworms (Koziol et al., 2016). Within the cortex of the adult tapeworm, muscle, germinative cells and neurons are tightly knit together in a close spatial association (Fig 1.5) (Halton and Maule, 2004; Koziol et al., 2010; Rozario and Newmark, 2015). This region can be described as a developmental 'signalling cylinder' (DSC). This project has shown that several factors from the Notch, Wnt and Hh pathways are expressed within the DSC. Hh and Notch factors are associated with the nervous system whilst Wnt factors are likely to be expressed in muscle cells. Together with our understanding of signalling and regeneration in planarians, a model can be proposed that links Notch, Hh and Wnt signalling in the DSC in the maintenance and differentiation of tapeworm tissues during homeostasis and strobilation. In this model, Notch and Hh signals (possibly together with *Hmic-Wnt5*) regulate neurogenesis by repelling aberrant axon growth. Hh signals are transported along nerve cords and induce Wnt signalling in muscle cells that then act as a blueprint to migrating germinative cells during strobilation and tissue turnover. Given that these pathways have been shown to be involved in cross-talk with each other (e.g. Espinosa et al., 2003; Hayward et al., 2005; Brack et al., 2008; Yazawa et al., 2009; Bertrand et al., 2012; Xie et al., 2013) and can be downstream signalling targets of each other, it is possible that they may be involved in a self-regulating feedback loop with each other.

7.7 Signalling pathways, proglottisation and embryogenesis

Proglottisation is a process that results in the serial differentiation of gonads and is used as means with which to massively increase fecundity (Stunkard, 1962). It evolved separately from strobilation (Olson and Caira, 1999; Olson et al., 2001, 2008) which refers to the external division of proglottides. Proglottisation results from the sequential proliferation of germinative cells in the medulla, that give rise to the genital primordia (Koziol et al., 2010). The expression of several factors (including *Hmic-Hh*, *Hmic-Ci*, *Hmic-Notch1*, *Hmic-Sfl* and some *Hmic-Fz*s) occurs centrally in the neck, and indicates that Hh, Wnt and Notch signalling are all involved in the proglottisation process. Signals emanating from the three pathways may be involved in the maintenance of germinative cells that give rise to proglottides. In mammals and *D. melanogaster*, the pathways are involved in the regulation of stem cell renewal and differentiation and are active in cancer stem cells (e.g. Reya et al., 2001; Hitoshi et al., 2002; Willert et al., 2003; Sato et al., 2004; Ohlstein and Spradling, 2007; Zhao et al., 2009). As such Wnt, Hh, and Notch signalling may also be involved in the maintenance and differentiation of stem cells that generate proglottides.

Factors from all three pathways are also expressed in the uterus. Notch, Hh and Wnt signalling are all involved in the patterning and specification of cell types during embryogenesis (reviewed in Yamaguchi, 2001; Pourquié, 2003; Varjosalo and Taipale, 2008; Martin and Kimelman, 2009; Petersen and Reddien, 2009; Niehrs, 2010). Expression of Wnt factors during

embryogenesis in planarians (Martín-Durán et al., 2010) suggests that Wnt factors, alongside those involved in Hh and Notch signalling, are also expressed during tapeworm embryogenesis, explaining expression within the uterus.

7.8 Developing a robust functional tool

There is a need to develop robust methods in model tapeworm systems with which to investigate gene function. Current functional methods in parasitic worms lag far behind those developed in free-living planarian systems (Sánchez Alvarado and Newmark, 1999) which are now used routinely. Even basic *in vitro* culture methods are problematic (reviewed in Koziol, 2016), in part due to their highly complex lifecycles. In the past, *H. microstoma* has been cultured *in vitro* (Evans, 1970, 1980) however, recent attempts at replicating these methods have proved ineffective (Pouchkina-Stantcheva et al., 2013; OlsonLab, unpublished). As such, this thesis provided the first steps towards a novel *in vivo* approach with which to investigate gene function. Whilst the early attempts described here are positive, confirmation is still needed and require future investigation. The biggest drawback to the injection system is that it is highly labour intensive, and thus low through-put (in part, this resulted in abandonment of these trials). Secondly, the precise dose of inhibitor (chemical or dsRNA) that oncospheres are exposed to cannot be regulated. As such, fine control of the system is unattainable and multiple biological repeats would be required. Despite these flaws, this is still a highly promising system that should be further developed.

Other tapeworm species have been cultured *in vitro* (Smyth, 1946; Smyth and McManus, 1989; Siles-Lucas and Hemphill, 2002; Jakobsen et al., 2012). However, these species have their own drawbacks. For example, the fox tapeworm *Echinococcus multilocularis* can be cultured *in vitro*, but is restricted to the study of larval development and culture of isolated cells (Norman and Kagan, 1961; Spiliotis and Brehm, 2009). As such, it is likely that gene function in tapeworms will need to be investigated concurrently in several species (including *H. microstoma*).

7.9 Addressing the aims of the thesis

The aims of this aims of this thesis were

1. To investigate candidate developmental pathways controlling tapeworm polarity and strobilation.
2. To develop and expand tools for investigating gene expression and function in the model tapeworm *H. microstoma*.

The Wnt, Hh and Notch pathways were investigated for their role in specifying the tapeworm AP axis and strobilation. Wnt (and possibly Hh) signalling plays a part in specifying the AP axis of tapeworms. In the case of the Wnt pathway, this is throughout development, in larvae, juveniles, adults and in individual segments. Wnt signalling is also involved in tapeworm strobilation, with the evolution of a potentially novel use of *Wnt11a*. Notch signalling may play a role in strobilation, so far it is not clear if it is involved in

the specification of segment polarity but it does not appear to play a role in determining overall AP polarity of the tapeworm.

In situ hybridisation (ISH) methods were expanded in *H*. *microstoma*. This included the optimisation of colorimetric ISH protocols and some of the first fluorescent ISH (FISH) and double FISH (dFISH) in this species. This has involved the synthesis of bench made fluorophores and the development of dFISH for the first time in *H*. *microstoma*. Secondly, the first steps towards the development of a novel *in vivo* approach in which to investigate gene function have been developed.

7.10 Future directions

Since starting this thesis, research into planarian developmental biology has rapidly expanded, generating vast amounts of data. Research into planarian systems have many advantages over their parasitic relatives. Importantly, functional tools have been established in planarians for many years now (Sánchez Alvarado and Newmark, 1999) and dFISH methods that are used as standard in planarians, have been developed for the first time in *H*. *microstoma* during the research carried out as part of this thesis. A wide range of cell markers, including those for neoblasts, muscle, neuronal cells have been developed in planarians (Reddien et al., 2005; Nishimura et al., 2010; Witchley et al., 2013). These markers need to be developed in *H*. *microstoma* and by doing so, will allow us to determine exactly what cell types are expressing factors including tapeworm PCGs and neural-related

genes including *Hmic-Hh* and *Hmic-Notch1*. The development of these markers should be the first priority as, unfortunately, we are probably still far away from developing a standardized *in vitro* protocol with which to investigate tapeworm gene function. Despite this, the *in vivo* approach to gene function in tapeworms, trialed as part of this thesis, still needs to be confirmed and is a highly promising avenue for investigating gene function. Finally, the study of gene expression (especially Wnt factors) in tapeworms with unusual body plans should prove highly informative in understanding the evolution of strobilation in tapeworms.

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Appendix 1

List of primers

Gene name	Model ID	Forward primer	Reverse primer
Hmic-Sfrp	HmN_000556500	TGGCCGATTGACTACTGCAGATG	TGATAGTGTTGGTAATAGGGATGCTGG
Hmic-Sfl	HmN_000359400	ACCTACTGTTCCCATGCAGC	TCCAAGCATCCCGTTGTTGA
Hmic-Wnt1	HmN_000328000	TGAGTGACAACGGAATCAAT	TCATAACTAGAGCGGCTTTC
Hmic-Wnt2	HmN_000112200	ATTTTACTCAAACACCCAACCGTC	GCATTTCACTTCACAACACCAAAC
Hmic-Wnt4	HmN_000808600	ACTGGCCGCCCGCGACCTGAACTG	TGGACCTTTTGAGTCAACACTACA
Hmic-Wnt5	HmN_000108100	TCCCTGCTAAAATGTTACCCATCA	CACAAAAGTTGGGTGAATCCTCAA
Hmic-Wnt11a	Not predicted	TTGTCGTGAAACAGGAGGCA	TGTGCCGTACTTCATTTCGGA
Hmic-Wnt11b	HmN_000022800	TGCTCTGCCTACTTCAGGTGGA	CGTGACCTCTTCCGCAGCACA
Hmic-FzA	HmN_000386300	TTCGAGGCAAGTTGATCCCC	CTTCGCCATCGCAGTTCATG
Hmic-Fz4/8	HmN_000319700	GCGGGCAAGAAATTCGCTAG	CTCGTGATGGCGACGACATA
Hmic-FzD	HmN_000227100	TGAAAATACCGTGCAGCCCT	TACCCTCCCGTGTTATCCGT
Hmic-FzE	HmN_000595200	TGACGAACCGATTCCAAGCA	GAGCAGGAGTTAGCCAAGCA
Hmic-Hh	HmN_000068600	TGCCCTCAGAAACCCACAACCT	ACGTCAACGTAGGGATGAACACCTGA
Hmic-Ci	HmN_000840400	GAAGGTGGCGGTGGGGCTTC	GATGCAGCAGCGGTGGTGGT
Hmic-Ptc	HmN_000602000	TGCCTAGGACTCAAGCTTGC	GCTCAATCGCATCGGTAGGA
Hmic-Smo	HmN_000930900	GCCCATACTGCCCTCACTTT	TTCCACGACGAGAACCACAG
Hmic-Sufu	HmN_000482400	AGCTTGCCATCGCCTATACC	ATAGGGAAAGGAGAGGGGCAT
Hmic-Fu	HmN_000686000	CTCGGCGCGTCTAGTGAGCG	AACAAGGCAGCGGGGACACG
Hmic-Slimb	HmN_000834500	GGAGACAAACGTTGTGTGGC	TGGGGTGATCGAGAGTGGAT
Hmic-Disp	HmN_000570400	TTCTGCAGCCGTTCAGTGAT	CCCAAACAGCATGGAGGGAT
Hmic-Notch1	HmN_000653600	CGATGCATCCGGACAGATGA	CGTTGTTCCAGTTGCTGTGG
Hmic-Notch2	HmN_000853800	TGGCTCCTCCCCCTGACAGC	GCATTATCCGTCCCGCCCCG
Hmic-Ser	HmN_000639400	CTGTTGCCCTGTCGGGCTGG	TGGGCTGGACGGTGTGGTCA
Hmic-Dl1	HmN_000055700	GCAACGCCACACAATGTCTT	TTCATAGGGTGGGGGAGGAG
Hmic-Dl2	HmN_000605500	AGAATGTGTGCTCGGCAAGA	TTGCAGAAAGGTTTGCGTGG
Hmic-Dl3	HmN_000714400	TACTGCAAATCCGACTTCAA	GATGGTGAGGTTTCTTCCTT
Hmic-Dl4	HmN_000734400	TGGCAGACTTCCCAATGGAC	AGTGACCCCCATTCAAGCAG
Hmic-Post2 +**T7** (T7 promoter site is shown in **bold**)	HmN_000187500	**TAATACGACTCACTATAGGGAG**AG GAGCCCTTTTGCGAGA	**TAATACGACTCACTAT**AGGGAGACGTG TGATTGGGGTTGA

Appendix 2

A-Tailing protocol

Reaction mix:

5x Promega buffer	5.0 µL
25 mM $MgCl_2$	2.0 µL
10 mM dATP	0.5 µL
Promega Taq (5U/µL)	0.5 µL
PCR H_2O	2.0 µL
Pre-cleaned DNA	15.0 µL
	25.0 µL

- Mix by vortexing and spin down
- Incubate at 72 ^{o}C for 30 min
- Clean using QIAquick PCR purification kit (Qiagen)

Appendix 3

Recipes for *in situ* hybridisation reagents

1x PBS: Dissolve 10 Phosphate buffered saline (PBS) tablets (Sigma) to 1 L dd H_2O. Adjust pH to 7.5. Diethyl pyrocarbonate (DEPC) treated and autoclaved

1x PBST: 1 L 1x PBS + 1% Tween 20

4% PFA/PBST: Dissolve 8 g Paraformaldehyde in 200 mL PBST

90% EtOH/PBST: 100 mL 1x PBST mixed with 900 mL 100% Ethanol

80% EtOH/PBST: 200 mL 1x PBST mixed with 800 mL 100% Ethanol

50% EtOH/PBST: 500 mL 1x PBST mixed with 500 mL 100% Ethanol

25% EtOH/PBST: 750 mL 1x PBST mixed with 250 mL 100% Ethanol

Proteinase K solution: Dissolve 0.125 g Proteinase K in 5 mL sterile, DEPC treated dd H_2O. Store at -20 ^oC

0.1 M TEA: Mix 6.65 mL of 7.53 M TEA stock in 493.35 mL autoclaved DEPC treated dd H_2O

10% CHAPS: Dissolve 5 g CHAPS in 40 mL sterile DEPC treated dd H_2O. Bring to 50 mL with sterile DEPC treated dd H_2O. Aliquot and store at -20 ^oC

50x Denhart's: 50x Denhardt's stock solution (Sigma)

10% Tween20: 10 mL Tween 20 mixed with 90 mL sterile DEPC treated dd H_2O

Heparin stock solution (100 mg/mL): Dissolve 0.5 g Heparin in 5 mL sterile dd H_2O. Aliquot and store at -20 ^oC

Yeast RNA 100 mg/mL: Dissolve 1 g total yeast RNA in 10 mL sterile dd H_2O. Aliquot and store at -20 ^oC

8 M LiCl: Dissolve 8.478 g LiCl in 15 mL dd H_2O. Adjust volume to 25 mL

20x SSC: Dissolve 175.3 g NaCl and 88.2 g Tri-sodium citrate in 800 mL DEPC treated H_2O. Adjust pH to 7.0. Adjust volume to 1L and autoclave

2x SSC + 0.1% Tween20: 100 mL 20x SSC, 890 mL sterile DEPC treated dd H_2O, 10 mL 10% Tween20

0.2x SSC + 0.1% Tween20: 10 mL 20x SSC, 980 mL sterile DEPC treated dd H2O, 10 mL 10% Tween20

1x MAB: Dilute 10x Maleic Acid Buffer solution (Roche) to a 1x solution

Hybridisation (-) buffer (Hyb-): 50 mL deionised Formamide, 25 mL 20x SSC, 0.1 mL Heparin stock, 2 mL 50x Denhardt's, 1 mL 10% Tween20, 1 mL 10% CHAPS, 2 mL 0.5 M EDTA pH 8.0, 17.9 mL DEPC treated dd H_2O. Store at -20 ^oC

Hybridisation (+) buffer (Hyb+): 99 mL Hyb(-), 1 mL yeast RNA (100 mg/mL). Store at -20 ^oC

Lamb serum: Heat inactivate at 60 ^oC for 30 min. Centrifuge at 10,000 rpm for 20 min at 4 ^oC. Aliquot and store at -20 ^oC

MAB + 2% BSA: Dissolve 10 g Bovine Serum Albumin (BSA) in 500 mL 1x MAB

Blocking buffer: 80 mL MAB + 2% BSA with 20 mL lamb serum

1 M Tris (pH 9.5): Dissolve 121.14 g Tris (hydroxymethyl) aminomethane in 800 mL dd H_2O. Adjust pH to 9.6. Adjust volume to 1 L with dd H_2O and autoclave

1 M $MgCl_2$: Dissolve 203.31 g MgCl in 800 mL dd H_2O. Adjust volume to 1 L and autoclave

5 M NaCl: Dissolve 292.2 g NaCl in 800 mL dd H_2O. Adjust volume to 1 L and autoclave

Alkaline phosphatase buffer: 10 mL 1 M Tris, 5 mL 1 M $MgCl_2$, 2 mL 5 M NaCl, 1 mL 10% Tween20, 82 mL sterile dd H_2O

50% Glycerol/PBST: 50 mL 1x PBST mixed with 50 mL 100% glycerol

80% Glycerol/PBST: 20 mL 1x PBST mixed with 80 mL 100 % glycerol

Appendix 4

Synthesis of bench-made TSA solutions*

Fluorescein tyramide conjugate synthesis protocol

1) Prepare DMFA-triethylamine solution by adding 15 µL of triethylamine to 1.5 mL of DMFA
2) Prepare tyramine solution by dissolving 1.5 mg of tyramine in 1.5 mL DMFA-triethylamine solution
3) Prepare Fluorescein-NHS ester solution by dissolving 50 mg NHS-Fluorescein in 5 mL DMFA
4) Mix Fluorescein-NHS ester solution and 1.37 mL tyramine solution and incubate in the dark at room temperature for 2 hours
5) Add 4.6 mL of 100% ethanol
6) Aliquot and store at -20^{o}C

Use at 1:100

Rhodamine tyramide conjugate synthesis protocol

1) Prepare DMFA-triethylamine solution by adding 10 µL triethylamine to 1 mL of DMFA
2) Prepare tyramine solution by dissolving 10 mg tyramine hydrochloride in 1 mL DMFA-triethylamine solution
3) Prepare Rhodamine-NHS ester solution by dissolving 25 mg rhodamine in 2.5 mL DMFA
4) Mix Rhodamine-NHS ester solution and 750 µL DMFA-triethylamine
5) solution in dark and leave at room temperature for 2 hours
6) Add 21.25 mL of 100% ethanol
7) Aliquot and store at -20^{o}C

Use at 1:500

* Protocols were kindly provided by Uriel Koziol (Universidad de la República de Uruguay, Uruguay) and Anna Protasio (Wellcome Trust Sanger Institute, UK).

Appendix 5

Automated dFISH Intavis run output

********** method-listing: dFISH medium baskets OLSON.IPM **********
********** path: C:\INTAVIS\Software\InsituPro VSi 3.1 cpr\Methods\Methods Whole mounts & vibratome sections\Medium baskets\
** 01.11.2016 - 16:26:27
** Software : InsituProVSi
** Version : 26.11.2015
********** configuration: InsituPro VSi-VT.IPC **********
********** path: C:\INTAVIS\Software\InsituPro VSi 3.1 cpr\

********** ROBOTER : 223v4.3.0 , ID: 10
Rinse
Home : X:275 Y:200 Z:0 F:0 0.1mm
Drain : X:280 Y:195 Z:300 F:300 0.1mm
Rinse : X:280 Y:520 Z:1080 F:1080 0.1mm

********** DILUTOR : 402 , ID: 0
Configuration
dilutor syringe : 10000
transfertubes : 10100 ul
transfertube - 2 by valve at ->
roboter output nr.: 3 nr
Pipetting
reservoir aspiration speed : 30 ml/min
prime (dispense) speed : 72 ml/min

aspiration speed LOW : 10 ml/min
aspiration speed MEDIUM : 15 ml/min
aspiration speed HIGH : 20 ml/min
dispense speed LOW : 10 ml/min
dispense speed MEDIUM : 15 ml/min
dispense speed HIGH : 20 ml/min

inside rinse vol. NORMAL : 700 µl
inside rinse vol. INTENSIVE : 1400 µl
outside rinse vol. NORMAL : 700 µl
outside rinse vol. INTENSIVE : 1400 µl
rinse dispense speed LOW : 20 ml/min
rinse dispense speed HIGH : 30 ml/min

(System: Baskets M)

********** THERMOCONTR 610 , ID: 1
Contacts
output cool-fan : 4 1..4

********** method ******************************
1 Module Starting Method
Tasks required for starting a method
1.1 XYZCheck
1.2 SetTempReg to : OFF
1.3 PrimeNeedle 12000µl
1.4 PrimePort 10000µl Port 1->Drain
Preparing Port 1
1.5 PrimePort 10000µl Port 2->Drain
Preparing Port 2
...
2 Module PBST Wash
2.1 IncubateVT 00:10 700µl PBST->Specimen 4x
...
3 Module Prot K max.batch:12 3.1
IncubateVT 00:05 700µl PBST->Specimen 2x
3.2 SetTempReg to : 20°C, <10min
3.3 IncubateVT 00:05 700µl Prot K->Specimen
3.4 SetTempReg to : RT
...
4 Module TEA Time
4.1 IncubateVT 00:05 700µl TEA->Specimen 2x
4.2 IncubateVT 00:03 700µl TEA low->Specimen 2x
4.3 IncubateVT 00:03 700µl TEA high->Specimen 2x
4.4 IncubateVT 00:05 700µl PBST->Specimen 4x
4.5 IncubateVT 00:30 700µl 4% PFA->Specimen
4.6 IncubateVT 00:05 700µl PBST->Specimen 5x
4.7 IncubateVT 00:10 700µl PBST->Specimen 2x
4.8 IncubateVT 00:05 700µl PBST->Specimen 2x
...
5 Module Prehybridisation
5.1 SetTempReg to : 60°C, <30min
5.2 IncubateVT 00:10 350µl Hyb-->Specimen
5.3 IncubateVT 00:10 700µl Hyb-->Specimen
5.4 IncubateVT 08:00 700µl Hyb+->Specimen
...
6 Module Probe Hyb
6.1 IncubateVT 12:00 700µl Probe->Specimen
6.2 IncubateVT 00:10 700µl Hyb+->Specimen 3x
6.3 IncubateVT 00:20 700µl 2x SSC->Specimen 4x
6.4 IncubateVT 00:30 700µl 0.2x SSC->Specimen 4x
6.5 SetTempReg to : RT
6.6 IncubateVT 00:15 700µl Washing buffer->Specimen 3x
...
7 Module Block & AB1
7.1 IncubateVT 00:30 700µl Blocking buffer->Specimen 4x
7.2 SetTempReg to : 4°C, <30min
7.3 IncubateVT 10:00 700µl AB1->Specimen
7.4 SetTempReg to : RT
7.5 IncubateVT 00:05 700µl Washing buffer->Specimen 4x
7.6 IncubateVT 01:00 700µl Washing buffer->Specimen 3x
...
8 Module TSA-FITC
8.1 IncubateVT 00:10 700µl PBST->Specimen 2x
8.2 IncubateVT 00:10 700µl PBS + Imidazole->Specimen 3x
8.3 IncubateVT 00:10 700µl TSA1 - FITC->Specimen
8.4 IncubateVT 00:05 700µl PBST->Specimen 5x
8.5 IncubateVT 00:10 700µl PBST->Specimen 2x
...

```
9                   Module          Quenching & Wash
9.1                 IncubateVT        00:20 700µl Sodium azide->Specimen  2x
9.2                 IncubateVT        00:05 700µl PBST->Specimen  4x
9.3                 IncubateVT        00:10 700µl PBST->Specimen  2x
...
10.1                IncubateVT        00:30 700µl Blocking buffer->Specimen  2x
10.2                SetTempReg          to : 4°C, <30min
10.3                IncubateVT        10:00 700µl AB2->Specimen
10.4                SetTempReg          to :  RT
10.5                IncubateVT        00:05 700µl Washing buffer->Specimen  4x
10.6                IncubateVT        01:00 700µl Washing buffer->Specimen  3x
...
11                  Module          TSA-TRITC
11.1                IncubateVT        00:10 700µl PBST->Specimen  2x
11.2                IncubateVT        00:10 700µl PBS + Imidazole->Specimen  3x
11.3                IncubateVT        00:10 700µl TSA2 - TRITC->Specimen
11.4                IncubateVT        00:05 700µl PBST->Specimen  5x
11.5                IncubateVT        00:10 700µl PBST->Specimen  2x
...
12                  Module          DAPI Staining
12.1                IncubateVT        00:10 700µl DAPI->Specimen
12.2                IncubateVT        00:05 700µl PBST->Specimen  6x
12.3                IncubateVT        00:10 700µl PBST->Specimen
...
13                  Module          Clearing
13.1                IncubateVT        00:10 700µl PBST->Specimen
13.2                IncubateVT        00:30 350µl 50% glycerol->Specimen
13.3                IncubateVT        01:00 700µl 50% glycerol->Specimen
13.4                Pause           REMOVE SPECIMEN CARRIER & PUT
PORT1 TUBE INTO BEAKER OF ddH2O
...
14                  Module          Finishing Method
Tasks required for finishing a method
14.1                RinsePort         10000µl Reservoir->Port 1
Cleaning Port 1
14.2                RinsePort         10000µl Reservoir->Port 2
Cleaning Port 2
14.3                PrimeNeedle         12000µl
14.4                SetTempReg          to :  OFF
...
...
********** end of method *** ******************
```

Appendix 6

Comparative analysis of Wnt expression identifies a highly conserved developmental transition in flatworms (2016)

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RESEARCH ARTICLE Open Access

Comparative analysis of Wnt expression identifies a highly conserved developmental transition in flatworms

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Abstract

Background: Early developmental patterns of flatworms are extremely diverse and difficult to compare between distant groups. In parasitic flatworms, such as tapeworms, this is confounded by highly derived life cycles involving indirect development, and even the true orientation of the tapeworm antero-posterior (AP) axis has been a matter of controversy. In planarians, and metazoans generally, the AP axis is specified by the canonical Wnt pathway, and we hypothesized that it could also underpin axial formation during larval metamorphosis in tapeworms.

Results: By comparative gene expression analysis of Wnt components and conserved AP markers in the tapeworms *Echinococcus multilocularis* and *Hymenolepis microstoma*, we found remarkable similarities between the early stages of larval metamorphosis in tapeworms and late embryonic and adult development in planarians. We demonstrate posterior expression of specific Wnt factors during larval metamorphosis and show that scolex formation is preceded by localized expression of Wnt inhibitors. In the highly derived larval form of *E. multilocularis*, which proliferates asexually within the mammalian host, we found ubiquitous expression of posterior Wnt factors combined with localized expression of Wnt inhibitors that correlates with the asexual budding of scoleces. As in planarians, muscle cells are shown to be a source of secreted Wnt ligands, providing an explanation for the retention of a muscle layer in the immotile *E. multilocularis* larva.

Conclusions: The strong conservation of gene expression between larval metamorphosis in tapeworms and late embryonic development in planarians suggests, for the first time, a homologous developmental period across this diverse phylum. We postulate these to represent the phylotypic stages of these flatworm groups. Our results support the classical notion that the scolex is the true anterior end of tapeworms. Furthermore, the up-regulation of Wnt inhibitors during the specification of multiple anterior poles suggests a mechanism for the unique asexual reproduction of *E. multilocularis* larvae.

Keywords: Antero-posterior axis, Cestodes, FoxQ2, Metamorphosis, Myocyte, Phylotypic, Planarian, Platyhelminthes, SFRP, Six3/6, Wnt

Background

Flatworms (Platyhelminthes) are a highly diverse and ubiquitous phylum of dorso-ventrally flattened animals that include a wide range of free-living and symbiotic forms. The majority of described species are obligate parasites belonging to the Neodermata, a monophyletic group that includes monogeneans, trematodes (flukes), and cestodes (tapeworms). The singular origin of neodermatan worms represents one of the most successful evolutionary transitions to parasitism seen in the animal kingdom [1–3].

The Neodermata have complex life cycles with one or more larval stages. Tapeworms (Cestoda) are particularly derived in their morphology and development, as they lack a gut and any trace of endoderm during embryogenesis [4]. The early embryonic development of tapeworms consists of asynchronous and asymmetric divisions, and there is no process resembling gastrulation [5–7]. The final product of embryogenesis is a highly reduced larval form, the oncosphere [7, 8]. This larva is passively taken up by an intermediate host, and is specialized for

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penetrating through the gut wall by means of six moving hooks and the secretions of penetration glands. The oncosphere metamorphoses into the next life stage, the metacestode, in a parenteral site of the first host. Most of the oncospheral cells are discarded, and the metacestode tissues are generated from a few stem cells that were set aside during embryogenesis [5, 8]. The metacestode consists of an anterior end, the scolex, which contains attachment organs, and a posterior undifferentiated body. Once the metacestode is ingested by the definitive host, it attaches to the wall of the gut, and develops into the adult form by continuously generating segments from the neck region behind the scolex. Within each segment, male and female reproductive systems develop, resulting in the generation of eggs by sexual reproduction.

Because of the unique morphology and development of tapeworms, it has been impossible to make meaningful comparisons with other flatworms, let alone other animal phyla. Even the true polarity of the tapeworm antero-posterior (AP) axis has been a matter of controversy [9, 10]. Conventionally, the scolex is taken to be the anterior of the adult, based on its functional orientation and on the centralization of the nervous system within it. However, it has been postulated that this accumulation of nervous elements could simply be due to the requirements for innervation of the muscular attachment organs of the scolex. Furthermore, the relative position of the male and female gonads in each segment (male gonads closer to the scolex) would be opposite that found in free-living flatworms if the scolex is considered anterior. Perhaps the most controversial point involves the relative polarity of the oncosphere and the adult; during metamorphosis, the scolex is formed at the pole of the oncosphere opposite to that containing the hooks (the "functional anterior end" of the oncosphere, Fig. 1), and therefore it has been postulated that a reversal of polarity occurs during this metamorphosis [8, 9].

It has recently been shown (free-living flatworms) that canonical Wnt/β-catenin signaling is involved in the specification and maintenance of the AP axis in planarians during regeneration and during normal tissue turnover, similar to what happens during early development in most bilaterian animals [11–15]. Specific Wnt ligands are expressed in gradients from the posterior end, and signaling through this pathway is required to maintain and specify the posterior of adult planarians. Conversely, extracellular inhibitors of Wnts are specifically expressed in the anterior end, and inhibition of canonical Wnt signaling is necessary for anterior specification.

We speculated that, although early development is very divergent between tapeworms and planarians, conservation of a fundamental AP specification program should be shared across the phylum. Thus, the later stages of planarian development should show homologous gene expression patterns to the oncosphere-to-metacestode metamorphosis in tapeworms, as these are the stages where the main body plan is established, whereas earlier developmental stages are specialized for their specific ecological requirements [8, 16].

In this work, we elucidate the expression patterns of Wnt ligands and inhibitors as well as other highly conserved animal markers of AP polarity during metacestode development in the tapeworms *Echinococcus multilocularis* and *Hymenolepis microstoma*. Our results show a striking conservation of gene expression, leading us to propose that these stages represent the phylotypic period of flatworms, and that the scolex is the true anterior end of tapeworms. Furthermore, our results indicate that the

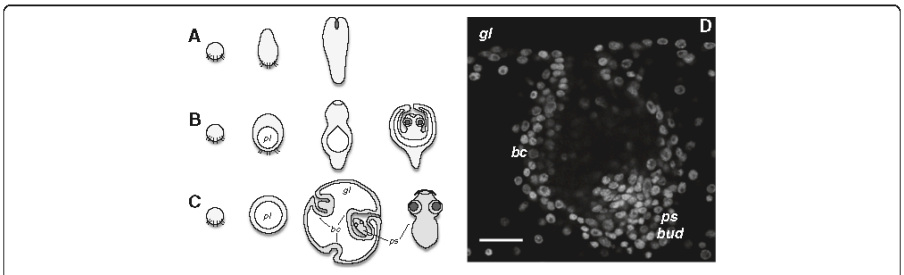

Fig. 1 The oncosphere to metacestode metamorphosis in different tapeworms. A generalized summary of development is shown for different tapeworm groups. **a** Development in basal eucestodes, without the formation of an inner cavity. **b** Development in most cyclophyllidean tapeworms, with the formation of a cavity (primary lacuna, *pl*) during early development. The scolex invaginates or retracts into this cavity during later development. **c** Development in *Echinococcus multilocularis*. The tissues surrounding the primary lacuna expand into the thin germinal layer (*gl*), from which many brood capsules (*bc*) invaginate. The protoscoleces (*ps*) develop within the brood capsules, and are released when the metacestode is ingested by the definitive host. **d** Nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) of an *E. multilocularis* metacestode vesicle from *in vitro* culture, showing the continuity of the germinal layer (*gl*), brood capsule (*bc*) and protoscolex bud (*ps bud*). Bar: 20 μm

unique development of the *E. multilocularis* metacestode is the result of a lack of AP polarity during early development, resulting in a completely posteriorized metacestode from which multiple foci of anterior development subsequently arise.

Results

A re-interpretation of *E. multilocularis* larval morphology and development

The life cycle outlined in the introduction is a generalization of the diversity found in the 'true' tapeworms, the Eucestoda (Fig. 1a). However, variations of this plan occur in particular groups. Many species of the order Cyclophyllidea form an internal cavity during early metacestode development, called the primary lacuna (Fig. 1b) [8, 17, 18]. The tissues surrounding this cavity form a cyst or bladder. The scolex retracts or invaginates into this cavity, and is protected by the surrounding tissue (Fig. 1b).

Among cyclophyllideans, metacestodes of *Echinococcus* spp. are the causative agents of dangerous zoonoses worldwide [19], and display unique development in their intermediate hosts (typically in the liver) (Fig. 1c) [17, 20]. Initially, only bladder tissue is generated from the oncosphere, forming a large vesicle that is filled with fluid, and contains only a thin layer of tissue in the periphery (the germinal layer) (Fig. 1c). From the germinal layer, secondary vesicles, called brood capsules, are formed towards the inner cavity. Within the brood capsules, nascent scoleces (protoscoleces) are formed by budding, resulting in massive asexual propagation (Fig. 1c). The protoscoleces are attached to the brood capsule by a thin stalk, and are released when the metacestode is ingested by the definitive host.

The evolutionary origin of the unique *E. multilocularis* metacestode remains unsolved. This is partly because the development of *E. multilocularis* has been historically regarded to be fundamentally different from that of other tapeworms, as the protoscoleces were considered to form within the central cavity, towards its interior (endogenous development), as opposed to forming on the external surface of the metacestode as in most other tapeworms (exogenous development) [8, 21–23]. However, we have recently shown, using confocal microscopy, that brood capsules and protoscoleces of *E. multilocularis* are actually formed from an invagination of the germinal layer of metacestode vesicles, and therefore just as in other tapeworms, the scolex is formed from the metacestode body wall towards the exterior [24] (Fig. 1c,d; see also [25, 26]). If one assumes that the scolex is the anterior end of tapeworms, then the *E. multilocularis* metacestode can be interpreted as showing differentiation along an AP axis, with many anterior ends (protoscoleces) followed along the AP axis by the brood capsules, and finally converging to one common posterior represented by the germinal layer of the vesicles. Therefore, we hypothesized that, during early development, the *E. multilocularis* metacestode is composed exclusively of posterior tissues and anterior development is suppressed. The remaining stages of metacestode differentiation are delayed, and only later do multiple foci of anterior development arise from the germinal layer. This is in contrast to most tapeworms, in which a single scolex forms very early during development, always in the region opposite to the hooks of the oncosphere [8].

Expression of Wnt and SFRP genes during early metacestode development of *E. multilocularis*

We analyzed the expression of homologs of Wnt ligands and of inhibitors of Wnt signaling involved in planarian AP specification using whole-mount in situ hybridization (WMISH) [11, 12, 14, 15, 27] (Figs. 2 and 3). Although tapeworms have lost many developmental genes conserved in most animals, they possess clear homologs of all families of Wnt ligands present in planarians [28, 29]. They also have one clear member of the SFRP family of Wnt inhibitors containing a cysteine-rich domain and a netrin domain [28], and another member with a divergent netrin domain dubbed SFRP-like. In planarians, three different SFRP genes are expressed in overlapping anterior domains of which *sfrp-1* is considered a *bona fide* marker of anterior specification during regeneration [11, 14, 27, 30].

Strikingly, the *E. multilocularis em-sfrp* and *em-sfl* genes are not expressed in the germinal layer of the metacestode (they cannot even be detected by RT-PCR), and expression first appears when brood capsule buds develop as small accumulations of cells protruding from the germinal layer (Fig. 2). Throughout the development of the brood capsules and the protoscoleces, *em-sfrp* is expressed in the anterior-most region, eventually becoming restricted to a few cells at the tip of the developing protoscolex (Fig. 2). *em-sfl* also shows anterior expression but is less restricted, with strong expression in the apical end of the protoscolex but also in the protoscolex body and in the brood capsule (Fig. 2). These genes are the earliest known markers of brood capsule development. Conversely, when we analyzed the expression of homologs of posterior Wnt genes from planarians (*em-wnt1*, *em-wnt11A* and *em-wnt11B*), they were all expressed in dispersed cells in the germinal layer of the metacestode vesicles, and during brood capsule and protoscolex development they were always expressed in posterior domains: *em-wnt1* and *em-wnt11B* were always restricted to the germinal layer and to the base of the brood capsule, whereas *em-wnt11A* was expressed throughout the germinal layer and brood capsule, and also at the posterior end of the developing protoscolex (Fig. 2). These results are not only compatible with our hypothesis, but also suggest that the formation of

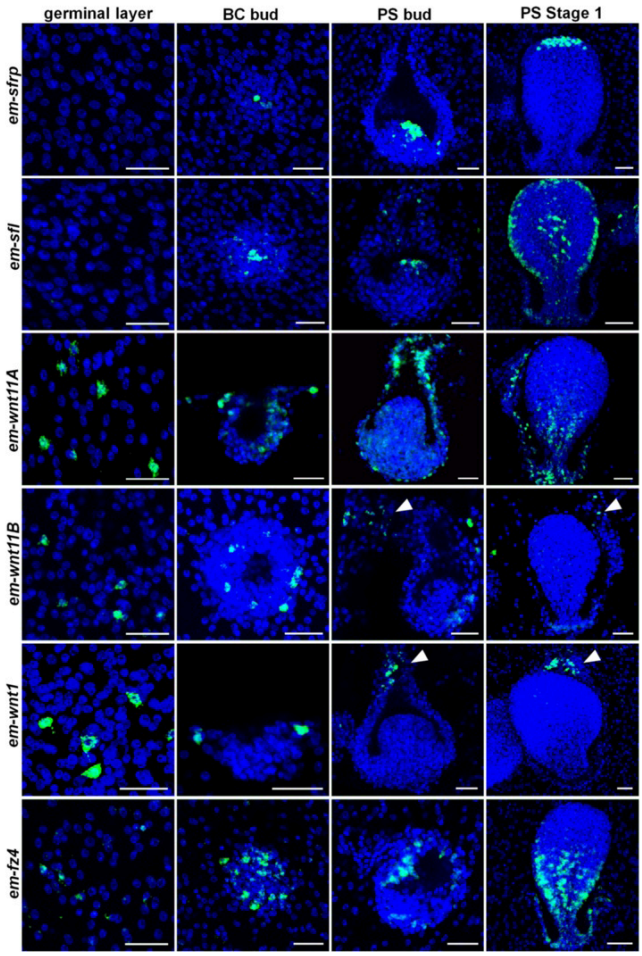

Fig. 2 Expression of SFRP, posterior Wnt, and Frizzled receptor genes during early *E. multilocularis* protoscolex development. Whole-mount in situ hybridization signal is shown in green and nuclear DAPI staining in blue. GL: Germinal layer; BC bud: Early brood capsule bud (shown from above for all genes except for *em-wnt11A* and *em-wnt1*, for which it is shown from a side); PS bud: Early protoscolex bud; PS stage 1: First stage of protoscolex development following the system of Leducq and Gabrion [25]. Arrowheads indicate positive cells at the base of the brood capsule. Bars: 20 μm

brood capsules is induced by the specific inhibition of posterior Wnt ligands that are widespread in the germinal layer. They also correspond remarkably to expression patterns in planarians, in which SFRP and Wnt genes are expressed in anterior and posterior overlapping domains, respectively, with *wnt1* being the most posterior [12, 27].

Encouraged by these results, we analyzed the expression patterns of the remaining Wnt genes in *E. multilocularis*

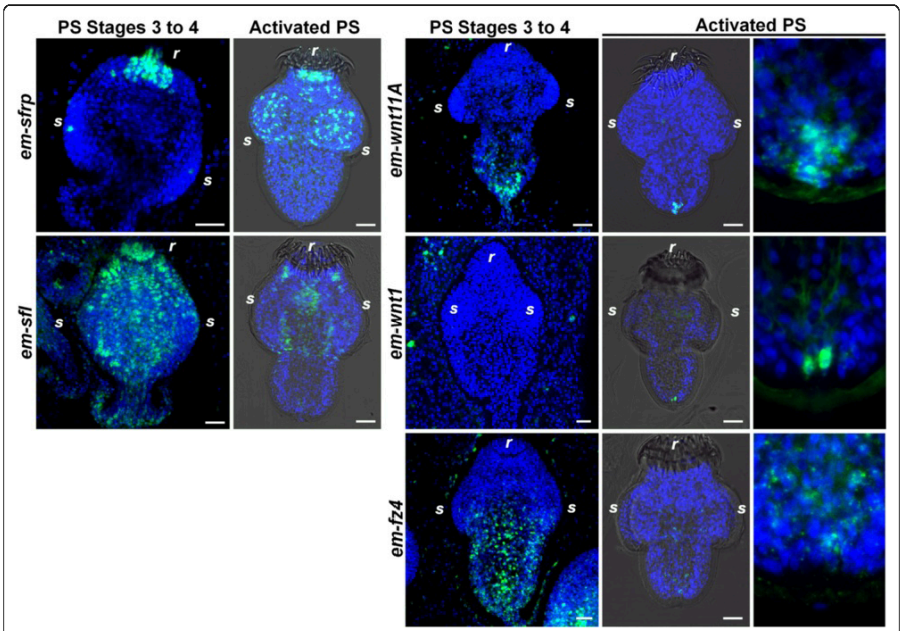

Fig. 3 Expression of SFRP, posterior Wnt, and Frizzled receptor genes during late *E. multilocularis* protoscolex development. Whole-mount in situ hybridization signal is shown in green and nuclear DAPI staining in blue. PS stages 3 to 4: Stages 3 to 4 of protoscolex development following the system of Leducq and Gabrion [25]. Activated PS: Activated protoscoleces after isolation from metacestode vesicles and successive treatments with pepsin and sodium taurocholate. *s*, Suckers; *r*, Rostellum. Bars: 20 μm

metacestodes (Fig. 4). The *wnt2* gene of planarians is expressed in two antero-lateral domains that surround the apical *sfrp1*$^{+}$ cells [14, 27]. We see a remarkably similar expression pattern of *em-wnt2*, since it is not expressed at all in the germinal layer or brood capsules, and expression appears in two antero-lateral domains of the developing protoscolex which surround the *em-sfrp*$^{+}$ expression domain. On the other hand, *wnt5* of planarians is expressed in lateral domains in planarians and is involved in the specification of the mediolateral axis through non-canonical Wnt signaling [27, 31]. Once again, we observe an equivalent pattern in the developing protoscolex, in which *em-wnt5* is expressed as two lateral stripes (Fig. 4). Unfortunately, we have not been able to obtain reproducible WMISH results for the remaining *wnt* gene, *em-wnt4*.

Finally, we analyzed the expression of *em-fz4*, an ortholog of a planarian Frizzled receptor expressed in the posterior-most region of the body and used as a specific posterior marker (named *fz4*, *fzT* or *fz-d* by different authors in different planarian species [11, 12, 32–34]) (Additional file 1). Frizzleds are a family of receptors for Wnt ligands that participate in both canonical and non-canonical signaling [35, 36]. The *em-fz4* gene is expressed in dispersed cells in the germinal layer, becomes strongly up-regulated during brood capsule development, and is always restricted to the posterior-most region of the protoscolex throughout development (Fig. 2).

Expression patterns during later *E. multilocularis* protoscolex development

We also analyzed the expression of these genes during later protoscolex development, including completely developed protoscoleces, which had been activated by mimicking the infection of the definitive host. For some genes, the expression patterns did not vary significantly (for example, *em-wnt2* and *em-wnt5*; Fig. 3). For *em-wnt11a*, expression was restricted to the posterior region of the protoscolex throughout, becoming progressively more restricted and only being expressed in a few posterior-most cells in the developed protoscolex (Fig. 3). The *em-wnt1* and *em-wnt11b* genes are always restricted

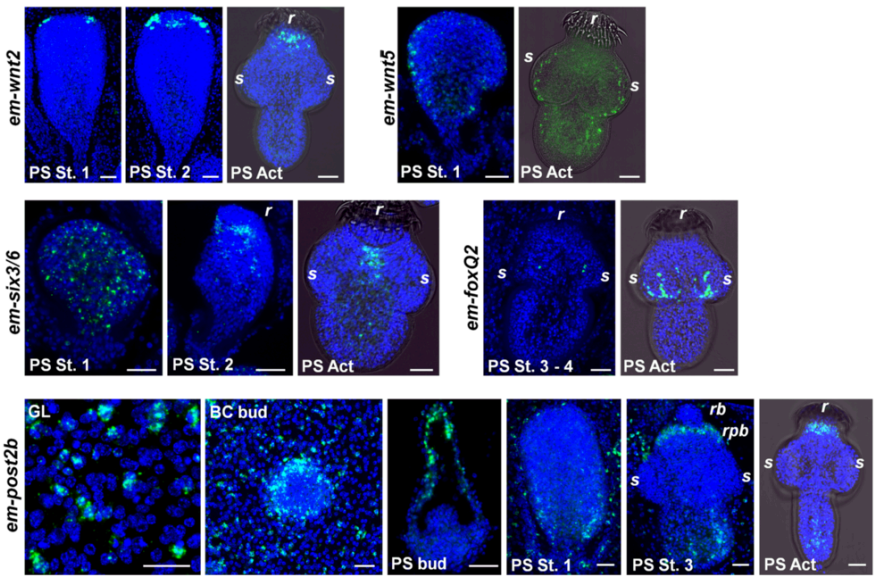

Fig. 4 Expression of other Wnt genes and AP axis markers during *E. multilocularis* metacestode development. Whole-mount in situ hybridization signal is shown in green and nuclear DAPI staining in blue. PS Act, Activated protoscoleces; PS st 1 and 2: First and second stage of protoscolex development following the system of Leducq and Gabrion [25]; GL: Germinal layer; BC bud: Early brood capsule bud (shown from above for all genes except for em-wnt11A and em-wnt1, for which it is shown from a side); *rb*, Rostellar bulb; *rpb*, Rostellar pre-bulb; *s*, Suckers; *r*, Rostellum. Bars: 20 μm

to the base of the brood capsule and are not expressed in the protoscolex itself (Fig. 3 and data not shown). Interestingly, after protoscolex release and activation, *em-wnt1* also becomes expressed in the posterior-most cells, similar to *em-wnt11a* (Fig. 3). Finally, *em-fz4* is always restricted to the posterior of the protoscolex and becomes barely detectable after development is complete (Fig. 3).

For other genes, the original expression patterns remained but additional sites of expression could be detected as development progressed. In the case of *em-sfrp*, the apical domain of expression persists throughout development, including in the apical rostellum (a muscular attachment organ containing hooks). However, during later development, new foci of expression appear in the scolex at each of the four developing sucker primordia, and expression remains in the suckers of the completely developed protoscolex (Fig. 3). In the case of *em-sfl*, it was later expressed in many tissues during protoscolex development, but was restricted to the tissues surrounding the rostellum in the completely developed protoscolex (Fig. 3).

In summary, expression patterns observed during early developmental stages are shown to be largely maintained in later stages, but new expression domains appear that are not directly comparable to those of planarians. This is compatible with the hypothesis that early metacestode metamorphosis is the most highly conserved stage of development, and developmental gene expression diverges in later stages as tapeworm-specific characters, such as the attachment organs, are formed.

Expression of conserved AP markers in *E. multilocularis*

In order to further test our hypothesis, we analyzed the expression patterns of AP markers that are well conserved in bilaterian animals but are not directly related to Wnt signaling. Our choices of markers were limited as many such genes have been lost in tapeworms or are too divergent to be identified unambiguously [29]. As a classical anterior maker, we have chosen the homeobox gene Six3/6 which is expressed in the anterior of bilaterian embryos (especially in the anterior-most region of the nervous system) [37]. In planarians, *six3/6* is expressed in

the outer and anterior-most region of the brain [38, 39]. A clear ortholog of *six3/6* is present in tapeworms (Additional file 2). In *E. multilocularis*, *em-six3/6* is not expressed in the germinal layer. Low levels of expression first appear throughout the early protoscolex buds, and the expression domain of *em-six3/6* is progressively restricted during protoscolex development to the region behind the developing rostellum (Fig. 4). This area represents the rostellar nerve ring, the most anterior region of the central nervous system [24]. We also analyzed the expression of an ortholog of *foxQ2* (Additional file 3), which is expressed in the anterior-most region of many animals [40–42]. However, the planarian ortholog is expressed in the brain but not in the anterior-most region [39], and similarly, in *E. multilocularis em-foxQ2* appears to be expressed in the nervous system of the scolex, but expression only occurs during late development in the region where the nervous system associates with the suckers (postero-lateral ganglia and sucker nerve rings [24]; Fig. 4).

Hox genes have conserved roles in the specification of body regions along the AP axis in bilaterian animals [43]. As a classical posterior marker, we chose the posterior Hox gene *post2*, a homolog of which is also expressed in the posterior body of planarians [15, 44]. The *E. multilocularis* ortholog *em-post2b* [45] is strongly expressed in the germinal layer and brood capsule, and is restricted to the posterior most regions of the protoscolex during early development, thus supporting our hypothesis (Fig. 4). At later stages of protoscolex development, a second expression domain also appears in the rostellar pre-bulb region, which forms the rostellar hooks. This expression domain becomes dominant in the completely developed protoscolex (Fig. 4).

Therefore, once again, we observe comparable gene expression patterns in early metacestode development of *E. multilocularis* and in adults of planarians. During later metacestode development, divergent expression patterns appear, which are related to tapeworm-specific morphological innovations with no clear counterpart in planarians.

Expression of Wnt and SFRP genes during *Hymenolepis microstoma* metamorphosis

Our results regarding gene expression in *E. multilocularis* strongly support our hypothesis that conserved gene expression patterns can be found during the larval metamorphosis of tapeworms. However, the development of *E. multilocularis* metacestodes is highly derived. Therefore, we wished to determine if similar gene expression patterns are also present in tapeworms with a more primitive form of development, and for this we chose the well-established model *H. microstoma* [46]. In *H. microstoma*, as in nearly all tapeworms, the oncosphere gives rise to a single juvenile worm with a scolex that develops at the pole opposite of the larval hooks (Fig. 1b). The polarity of the oncosphere is therefore reflected in the AP axis of the metacestode. However, like *E. multilocularis* and most other cyclophyllidean tapeworms, a primary lacuna (i.e. cavity) forms [47], later collapsing and encysting the nascent tapeworm.

Clear orthologs of all the described Wnt and SFRP genes of *E. multilocularis* are present in *H. microstoma*, and were identified with the same name, together with an *hm* prefix. As early as 48 hours after ingestion by the intermediate host, expression of the Wnt inhibitor *hm-sfrp* appears at the pole that will give rise to the scolex and is maintained throughout metamorphosis (Fig. 5). The *hm-sfl* gene is initially expressed apically, and later extends along the anterior region in two lateral stripes that begin sub-apically and end short of the opposite pole. In both cases, expression domains mirror those of *E. multilocularis* during protoscolex formation within the brood capsules (Fig. 2). Expression of 'posterior' Wnts *hm-wnt1*, *hm-wnt11a* and *hm-wnt11b*, and the frizzled receptor *hm-frzd4* is largely ubiquitous at the onset of metamorphosis, but becomes increasingly restricted to the posterior as the cells of the apical pole proliferate and condense to form the anterior regions. In addition, Wnts *hm-wnt11a* and *hm-wnt11b* show strong expression in two cells that mark the posterior pole, and *hm-wnt11a* also shows lateral expression in the central portion of the larvae (Fig. 5 and Additional file 4). Discrete expression of *hm-wnt11a* and *hm-wnt11b* at the posterior pole in *Hymenolepis* mirrors that seen in *E. multilocularis* during protoscolex development (Fig. 3). Thus, like in *E. multilocularis*, the metamorphosis of the *H. microstoma* larva begins with ubiquitous expression of Wnt ligands, from which a pole of Wnt inhibition leads to anterior development. The expression of genes coding for posterior Wnt ligands further supports the homology of the *E. multilocularis* germinal layer and the tissues that encyst the *Hymenolepis* juvenile. Other Wnt genes also replicate the expression domains observed in *E. multilocularis*: *hm-wnt2* is initially expressed in two anterolateral foci, which later expand slightly to surround the developing rostellum, whereas *hm-wnt5* is expressed in the lateral margins throughout larval development (Fig. 6 and Additional file 5). Taken together, the expression domains of these genes corroborate the expression of Wnt components seen in both tapeworm and planarian flatworms. They also help to confirm homologies between the convoluted morphology of *E. multilocularis* larvae and more typical tapeworm larval forms.

Muscle cells are a source of Wnts in the *E. multilocularis* germinal layer

In planarians, Wnts, SFRPs, and other genes related to the specification of the body axes (the so-called 'position control genes', PCGs) are expressed by muscle cells in the body wall, providing positional information during

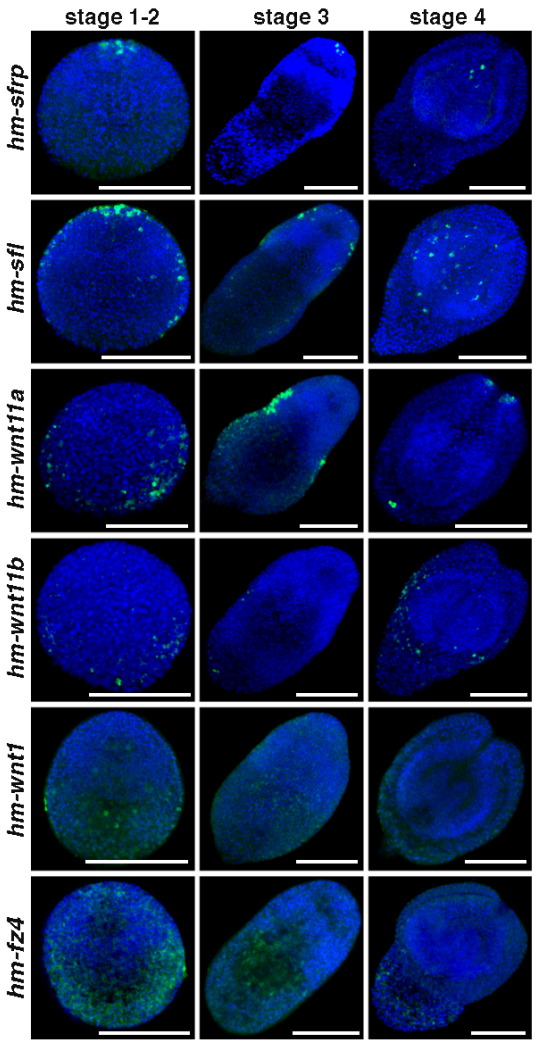

Fig. 5 Expression of SFRP and 'posterior' Wnt genes during *H. microstoma* metacestode development. Staging of larvae follows Voge [47]. Whole-mount in situ hybridization signal is shown in green and nuclear DAPI staining in blue. Bars: 50 μm

normal tissue turnover and regeneration [48]. The products of PCGs influence the behavior of neoblasts, which are pluripotent stem cells that do not express PCGs.

In tapeworms, similar stem cells (often called germinative cells) exist [49]. We have recently characterized the stem cells of *E. multilocularis*, and demonstrated that

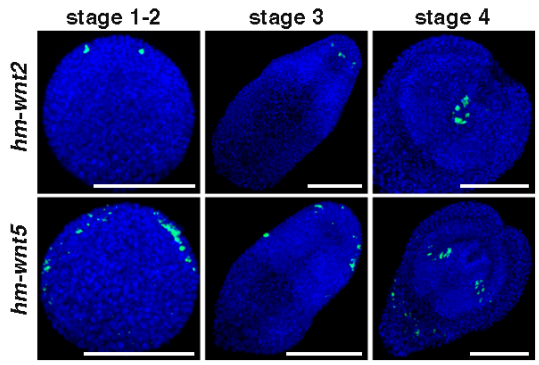

Fig. 6 Expression of other Wnt genes during *H. microstoma* metacestode development. Staging of larvae follows Voge [47]. Whole-mount in situ hybridization signal is shown in green and nuclear DAPI staining in blue. Bars: 50 μm

these cells are the only proliferative cells, as is the case in all flatworms studied to date [50]. Therefore, they can be specifically labeled by incubation with thymidine analogs such as 5-ethynyl-2'-deoxyuridine (EdU), which are incorporated into DNA during replication [50]. In order to determine if Wnt and SFRP genes are expressed by stem cells, we performed double labeling of WMISH for each gene together with the detection of EdU incorporation. We observed little to no incorporation of EdU in cells positive for *em-wnt1* (0.0 %; n = 148 cells), *em-wnt11a* (2.2 %; n = 716 cells), and *em-wnt11b* (0.8 %; n = 241 cells) in the germinal layer (Fig. 7). This was also the case in developing brood capsules and protoscoleces for *em-wnt1* (0.3 %; n = 377 cells), *em-wnt11a* (1.6 %, n = 1,150 cells), *em-wnt11b* (0.7 %; n = 241 cells) and *em-sfl* (0.2 %; n = 426 cells) (Fig. 7). Furthermore, *em-sfrp* is only expressed in the post-mitotic apical region [50]. Therefore, as in planarians, there is no significant expression of PCGs in tapeworm stem cells.

In the germinal layer of *E. multilocularis* vesicles, there are muscle cells that form a layer of disorganized muscle fibers. In tapeworms, the contractile muscle fibers (myofibers) are only connected by thin cytoplasmic strands to the main cell body (myocyton) containing the nucleus and most organelles [51]. We performed immunohistofluorescence of metacestode vesicles with an anti-tropomyosin (TPM) antibody that specifically labels the muscle fibers of tapeworms, including those of *E. multilocularis* [50, 52, 53]. Besides the strong signal in large muscle fibers, in many cases we could also observe thin TPM^+ filaments in the cytoplasm of myocytons that converged into myofibers (Fig. 8). By double labeling, we observed that many cells expressing *em-wnt1* and *em-wnt11a* in the germinal layer are muscle cells, since they also have TPM^+ filaments in the surrounding cytoplasm, and the $WMISH^+$ cytoplasm was clearly connected to long TPM^+ myofibers (Fig. 8). Because not all muscle cells showed TPM^+ fibers in the myocyton, we were unable to determine the percentage of muscle cells expressing each gene. Therefore, posterior Wnts are not only expressed in similar domains in *E. multilocularis* metacestodes and planarians adults, but also by the same cell type.

Discussion

The polarity of the tapeworm AP axis is conserved with other bilaterians

Our results clearly support the classical assumption that the scolex represents the true anterior end of tapeworms, and suggest that the condensation of the nervous and osmoregulatory systems present in the scolex are examples of cephalization. One of the main historical controversies regarding the AP axis of cestodes has been the interpretation of the polarity of the oncosphere, and how it relates to the polarity of the metacestode and adult [8]. The pole containing the hooks and the pores of the penetration glands has been considered functionally anterior, but the scolex develops from the opposite pole. However, oncospheres lack a brain (even the presence of nerve cells is controversial), do not move directionally and are taken up passively by the host [8, 54]. The most basal cestode lineages (Amphilinidea and Gyrocotylidea) have a more complex ciliated larva with ten hooks called the lycophora. This larva has an anterior brain and penetrates actively through the body wall of the intermediate host [55]. Importantly, during swimming the end of the lycophora bearing the hooks is directed posteriorly [56]. In the amphilinidean *Austramphilina elongata*, once the lycophora larva makes contact with the host with its anterior

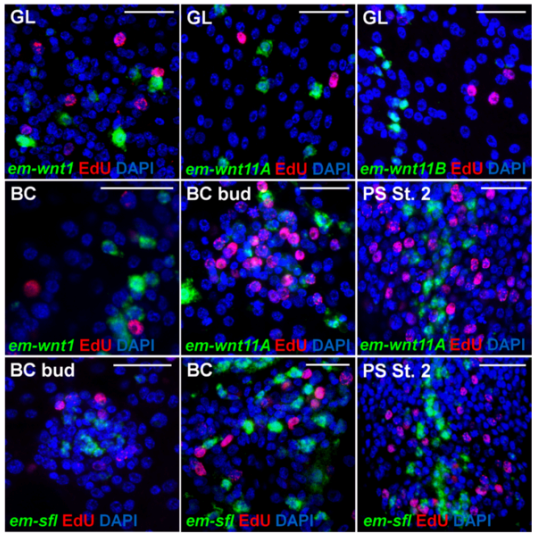

Fig. 7 Lack of EdU incorporation in cells expressing Wnt and SFRP genes in *E. multilocularis* metacestodes. Green: Whole-mount in situ hybridization (WMISH); red: EdU detection; blue: nuclear DAPI staining. EdU labeling was done by culturing metacestodes *in vitro* for 5 hours with 50 μM EdU. Notice the lack of co-localization between WMISH and EdU for each gene at each stage. GL: Germinal layer; BC bud: Early brood capsule bud (shown from above for all genes except for em-wnt11A and em-wnt1, for which it is shown from a side); PS st 2: Second stage of protoscolex development following the system of Leducq and Gabrion [25]. Bars: 20 μm

end, it bends into a U-shape that brings the posterior hooks and the anterior end together as the hooks participate in the penetration of the host [55]. During development of the juvenile, the end opposite of the hooks develops into a sucker for attachment [55]. Therefore, in basal cestodes, the functional end of the larva is the same as that of the adult, and the hooks are simply posterior organs for attachment and penetration. Wnt expression corroborates this notion and thus supports an evolutionary transition in which the oncosphere was derived by extensive reduction of a lycophora larva, and the posteriorly-oriented hooks maintained their role for penetration (now through the gut of the host) as eucestodes transitioned to a passive mode of infection of the intermediate host.

Another classical argument opposing the view that the scolex is the anterior pole of tapeworms is the fact that, if the scolex is considered anterior, then the relative position of the male and female gonads (testes anterior to the ovary) would be opposite that found in free-living flatworms. It is therefore possible that a change in the relative position of the gonads in the adult (that is, after the developmental stages covered in this work) occurred during tapeworm evolution. Finally, when the scolex is considered anterior, tapeworms show a form of growth that is contrary to that of all other segmented animals, as new segments are formed in the anterior germinative region in the neck (as opposed to a posterior growth zone as seen, for example, in annelids) [10]. However, tapeworm segmentation evolved *de novo* in the eucestode lineage and is not homologous to that seen in other animals [3, 57], making developmental comparisons impossible.

A phylotypic stage for flatworms

The Neodermata and several derived groups of free-living flatworms have extremely divergent modes of early embryonic development as a consequence of their unique eggs, which contain not only the oocyte, but also specialized cells called vitellocytes that produce yolk and shell proteins [2, 58]. Different flatworm groups have developed independent solutions to the problem of incorporating the yolk mass into the embryo, resulting in unique early developmental patterns. This is in stark contrast to the

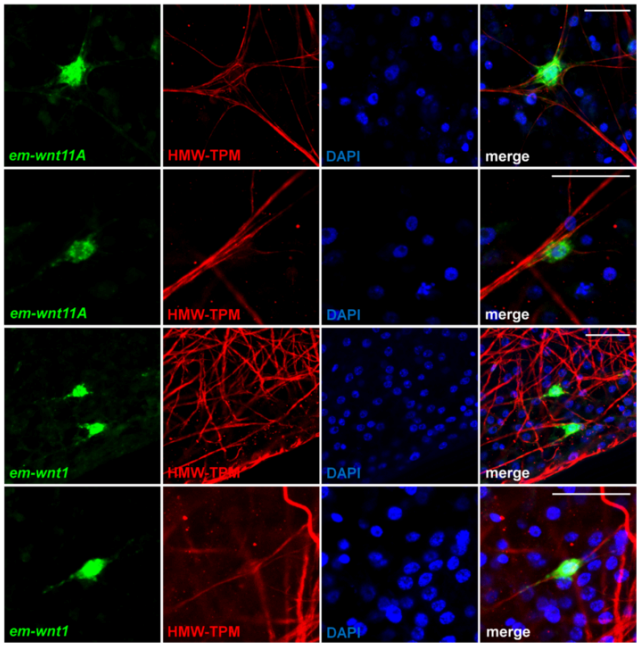

Fig. 8 Expression of Wnt genes in muscle cells in *E. multilocularis* metacestode vesicles. Green: Whole-mount in situ hybridization; red: immunodetection of high molecular weight tropomyosins; blue: nuclear DAPI staining. For each gene, two examples of positive muscle cells with different morphologies are shown. Bars: 20 μm

spiral cleavage present in most basal flatworm lineages and also in many other members of the superphylum Lophotrochozoa [58]. Therefore, it is very difficult to search for homologies in the development of different flatworm groups based solely on morphological grounds.

Despite this morphological diversity, we observe that the expression patterns of genes related to AP polarity and other PCGs are strikingly similar in tapeworms during larval metamorphosis and in planarians at late stages of embryonic development (Fig. 9). In contrast, early embryonic stages are highly divergent, and represent specific adaptations to their unique lifestyles. In planarians, early blastomeres disperse into the mass of vitellocytes (in a process aptly named 'blastomere anarchy') and eventually surround it. Later, a transient embryonic pharynx is formed [16]. All of these modifications are a consequence of the need to incorporate the external yolk, arising as an evolutionary adaptation to competition between several embryos within a single egg for limited yolk resources [16, 58]. During early development of planarians, many PCGs are already expressed, but in patterns that do not resemble those in later stages or in other animals, and the relationships between the axes of the early embryo and the adult are not clear [59]. At later stages of development, the definitive regions and tissues of the body of the embryo arise from ventral condensations of blastomeres, and it is at this stage that the PCGs acquire canonical expression patterns [59]. A similar developmental stage has been described in other flatworm groups, and it has been proposed to be the phylotypic stage for free-living flatworms [58]. The phylotypic stage is usually defined as a stage during mid-embryogenesis where the similarity in body plan and corresponding expression of developmental genes is maximal across the species of a phylum, and the definitive body plan of the animal is established [60, 61]. The phylotypic stage is expected to be highly conserved due to developmental constraints of numerous global interactions (unlike early

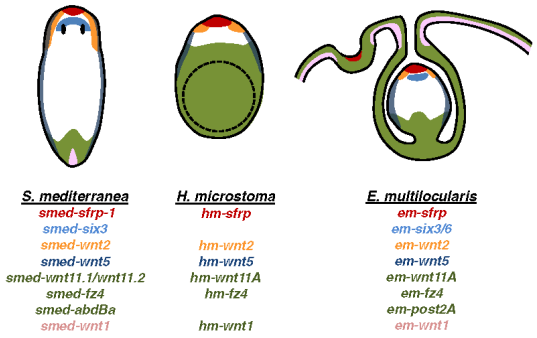

Fig. 9 Comparison of gene expression patterns in adults of the planarian *Schmidtea mediterranea*, with *H. microstoma* and *E. multilocularis* metacestodes. Approximate expression domains are shown with different colors, and in some cases several genes with slightly different expression domains were grouped together. References for the *S. mediterranea* expression patterns are given in the main text

development, which is highly variable in many taxa) and significant similarities can also be drawn at this stage with gene expression patterns in other animal phyla [60, 61].

We propose that the phylotypic stage suggested for free-living flatworms also applies to tapeworms. In the case of tapeworms, early development is also divergent, and the formation of the oncosphere is an evolutionary adaptation for the infection of the first host of the life cycle. The metacestode stage can be considered to have the basic body plan of tapeworms, as in basal cestode groups the only further development that occurs is sexual maturation, and segmentation in eucestodes is an evolutionarily derived novelty imposed over this basic body plan [3, 55, 57]. Therefore, during early metamorphosis of the oncosphere, the body plan is established and it is precisely at this stage that strong conservation is seen in the expression patterns of metazoan developmental genes. Later, development again diverges between groups, as specific organs of attachment are formed, representing the onset of the next ontogenetic phase of the life cycle. The expression of some PCGs diverges at this point, suggesting that they have become exapted for novel roles, in agreement with the idea that only the early metamorphosis represents the phylotypic stage. It would be interesting to determine the expression of PCGs in early tapeworm embryogenesis, but this is technically challenging as the embryo develops within the egg, and in cyclophyllideans also *in utero*.

In planarians, the anterior pole of the head has been proposed to organize anterior development during adult regeneration and expresses *sfrp-1* together with other inhibitors of Wnt signaling [62]. An apical pole of *sfrp-1* expression is also the earliest expression pattern of a PCG in the embryo that resembles that found in the adult [59]. Similarly, an apical pole of *sfrp* expression is observed very early during tapeworm anterior development. Interestingly, this region later becomes the rostellum, and continues to express *sfrp*. The rostellum is an evolutionary modification of the apical organ, an attachment organ that is formed in all tapeworms during metamorphosis and which may persist in the adult or be only transitory [4, 18]. Because of the universal conservation of this apical organ in tapeworms, it is possible that it also has a conserved role as an anterior organizer and, in fact, such a role has been previously proposed for the rostellum [63].

Evolutionary origins of the unique *E. multilocularis* development

The massive metacestodes of *Echinococcus* spp. result from extensive growth of the germinal layer. In the related metacestodes of *Taenia* spp. (that together with *Echinococcus* spp. comprise the family Taeniidae), the bladder also reaches large proportions, and one or many scoleces develop from the bladder tissue. The morphology of the metacestodes from *Taenia* spp. can be remarkably varied, including large bladders with a single scolex (cysticercus), with many invaginated scoleces (coenurus), or with many externally protruding scoleces ('polycephalic larvae') [17, 64, 65], but in all cases the scolex develops towards the exterior of the bladder (exogenous development). Asexual formation of many scoleces is found in many basal taeniid lineages, suggesting that it was present in the last common ancestor of the family, but homology of asexual reproduction in taeniids is controversial [65–69]. Because the development of *E. multilocularis* is actually also exogenous, we propose that there is no fundamental difference in the development of

taeniid metacestodes, supporting the hypothesis that asexual scolex formation is ancestral in taeniids (and was lost secondarily in several lineages). One unique characteristic of *E. multilocularis* metacestodes is the secretion of a thick external acellular covering, the laminated layer [70]. The brood capsules form as invaginations of the germinal layer that are not accompanied by the laminated layer. Therefore, brood capsules can be regarded as specializations of the germinal layer in which protoscoleces can develop without a laminated layer cover.

The *E. multilocularis* metacestode initially forms as a hollow cyst that lacks morphological polarity, and shows widespread expression of posterior Wnt genes. This suggests that, in *E. multilocularis*, anterior development is suppressed during the early stages of the oncosphere-to-metacestode transition, and that Wnt signaling is important for maintaining the posterior identity of the tissues. The formation of protoscoleces only occurs after months of infection [21, 71], and is accompanied by the expression of Wnt inhibitors, indicating that suppression of Wnt signaling is important for the formation of anterior structures. In contrast, in other tapeworms, the scolex primordium develops very early (for example, at 48 hours post-infection in *H. microstoma*) as a condensation of cells that always forms opposite the oncospheral hooks [8]. In classical studies of *E. multilocularis* metamorphosis, there is no indication of early anterior development and later regression, suggesting that anterior development is suppressed from the beginning [21]. Scattered studies of the development of metacestodes of the related *Taenia* spp. also indicate that they initially lack AP polarity, and develop as bladders from which one or more scolex primordia appear later [63, 72–76]. The lack of AP polarity during initial metacestode development, leading to a lack of correspondence between the polarity of the oncosphere and that of the metacestode, may be a characteristic of all taeniids.

Finally, the presence of muscle fibers in *E. multilocularis* metacestode vesicles has been enigmatic, since the fibers are disorganized and the vesicles lack motility [21, 24, 77]. The muscle system is probably homologous to that found in the bladder of other taeniids: in *Taenia* spp., the bladder tissue also has muscle fibers, is motile, and may assist in the evagination of the scolex [17, 24, 78]. Herein, we show that *E. multilocularis* muscle cells are a source of Wnt ligands, and suggest that this may account for the retention of this cell type in the otherwise immobile metacestode.

Conclusions

The unique biology of tapeworms has been a source of fascination and speculation, but their uniqueness has also confounded our ability to compare them to other animals and has hampered our understanding of their evolutionary origins. Identification of a conserved developmental stage in tapeworms opens the possibility to perform meaningful comparisons with free-living flatworms and other phyla for the first time. Conservation of the flatworm stem cell system and of underlying gene regulatory networks suggests that reciprocal illumination will come from studies of both planarians and parasitic flatworms [79].

Methods

Maintenance, culture and collection of tapeworms

E. multilocularis isolates were maintained by serial intraperitoneal passage in *Meriones unguiculatus* as previously described [80]. The isolates used were GH09 and J2012, obtained from accidental infections of Old World Monkeys in a breeding enclosure [81], and MS10/10, obtained from an infected dog. All isolates had been passaged for 5 years or less at the time of this study. Animal experiments were carried out in accordance with European and German regulations on the protection of animals (Tierschutzgesetz). Metacestode vesicles obtained from *in vivo* culture were co-cultured *in vitro* with rat Reuber hepatoma cells as previously described [80]. After at least 2 months of *in vitro* culture, metacestode vesicles began the development of brood capsules and protoscoleces, and were collected and fixed for WMISH and immunohistofluorescence with 4 % paraformaldehyde prepared in phosphate buffered saline following the method of Koziol et al. [50]. Completely developed protoscoleces were isolated from parasite material maintained *in vivo*, and activated by successive treatments with pepsin at low pH and sodium taurocholate [82].

The Nottingham strain of *H. microstoma* [46] was maintained *in vivo* using outbred BALB/c mice and flour beetles (*Tribolium confusum*). Infection of beetles was produced by exposing them for 24 hours to macerated gravid tissues of *H. microstoma*. Then, beetles were allowed to feed on flour and metacestodes at different stages of metamorphosis were obtained by dissection on successive days. Larvae were fixed with paraformaldehyde prepared in phosphate buffered saline, as previously described [50].

Molecular cloning

Genes of interest were identified by BLAST searches against the genomes and derived gene models of *E. multilocularis* and *H. microstoma* [29] followed by phylogenetic analyses (Additional files 1, 2 and 3). Fragments of the coding domain sequences of *E. multilocularis* genes were amplified by RT-PCR using pools of cDNA from *in vitro* cultured metacestode vesicles, primary cells and protoscoleces, and cloned into pDrive (Qiagen, Hilden, Germany) or pJet1.2 (Thermo Scientific, Schwerte, Germany) following standard procedures, as previously described [50].

Hymenolepis transcripts were amplified using cDNA from larval or adult specimens and cloned into StrataClone vectors (Agilent Technologies, La Jolla, CA, USA). Colonies were picked and checked for insert directionality via PCR, then used as templates for PCR amplification using M13 primers, resulting in products with T3/T7 promoter sites that were purified and used subsequently as templates for reverse transcription. A list of all gene model codes and primers used in this work is provided in Additional file 6.

Whole-mount in situ hybridization (WMISH)

Tyramide-FITC-based fluorescent and alkaline phosphatase-based conventional WMISH of *E. multilocularis* and *H. microstoma* larval stages were performed with digoxigenin-labeled antisense probes, as previously described [50]. *E. multilocularis* samples were analyzed by confocal microscopy using a Leica TCS SP5 (Leica Microsystems, Wetzlar, Germany). All gene expression patterns reported in this work from *E. multilocularis* metacestodes were obtained from at least two different WMISH experiments, starting from independent batches of *in vitro* cultured animals, with at least five metacestode vesicles analyzed in each experiment. Those of *Hymenolepis* are based on multiple independent assays, each containing approximately 10 *in vivo*-reared specimens of each larval stage (approximately 50 larvae per tube). Alkaline phosphatase-based WMISH specimens were imaged using differential interference contrast microscopy on a Leica DM5000B light microscope. Fluorescent specimens were imaged using a Nikon A1 confocal microscope and maximum projections created using ImageJ v. 2 [83]. Control experiments using labeled sense probes were always negative (data not shown).

In vitro labeling with 5-ethynyl-2′-deoxyuridine (EdU)

In vitro labeling with 50 μM EdU was done for 5 hours and fluorescent detection with Alexa Fluor 555 azide was performed after WMISH following the method described in by Koziol et al. [50].

Immunohistofluorescence

The immunohistofluorescence procedure described by Koziol et al. [24] was carried out after WMISH detection. Anti-HMW-tropomyosin [53] was used as primary antibody at a 1:500 dilution. The secondary antibody was anti-rabbit conjugated to tetramethylrhodamine (Jackson ImmunoResearch, West Grove, PA, USA).

Additional files

Additional file 1: Phylogeny of Frizzled receptors. Frizzled receptors from *H. sapiens*, *D. melanogaster*, the trematode *S. mansoni* [84], the planarian *S. mediterranea* [84], and the tapeworms *E. multilocularis* and *H. microstoma* [29] were aligned, and a phylogeny was estimated by Maximum Likelihood analysis (with a JTT model) using MEGA 5.2 [85]. The tree was rooted using the related Smoothened receptors. Bootstrap support values from 1,000 replicates are indicated next to the nodes. Nodes with lower than 50 % support were collapsed. Genbank accession codes are given for *H. sapiens* and *D. melanogaster*; GeneDB accession codes are given for *E. multilocularis* and *H. microstoma*; gene names from [84] are given for *S. mansoni* and *S. mediterranea*. The *fz4* genes of *E. multilocularis* and *H. microstoma* are indicated by arrows. (PDF 9 kb)

Additional file 2: Phylogeny of sine oculis-homeobox (SIX-HD) transcription factors. The homeodomain (HD) and SIX domains of SIX-HD proteins of *H. sapiens*, *D. melanogaster*, *E. multiloculars*, and *H. microstoma* were aligned together with published planarian SIX-HD proteins, and a phylogeny was estimated by Maximum Likelihood analysis (with a JTT model) using MEGA 5.2 [85]. Bootstrap support values from 1,000 replicates are indicated next to the nodes. Genbank accession codes are given for *H. sapiens*, *D. melanogaster*, and for the planarians *S. mediterranea* and *G. tigrina*; GeneDB accession codes are given for *E. multilocularis* and *H. microstoma*. Different groups of SIX-HD proteins are outlined, and the *six3/6* genes of *E. multilocularis* and *H. microstoma* are indicated by arrows. (PDF 109 kb)

Additional file 3: Phylogeny of Forkhead (FOX) transcription factors. The FKH domain of forkhead proteins of *H. sapiens*, *D. melanogaster*, *E. multilocularis*, *H. microstoma*, and published FoxQ2 homologs from other animals were aligned, and a phylogeny was estimated by Maximum Likelihood analysis (with a JTT model) using MEGA 5.2 [85]. Bootstrap support values from 1,000 replicates are indicated next to the nodes. Nodes with lower than 50 % support were collapsed. GeneDB accession codes are given for *E. multilocularis* and *H. microstoma* and Genbank accession codes are given for all other sequences. The FoxQ2 group is outlined, and the *foxQ2* genes of *E. multilocularis* and *H. microstoma* are indicated by arrows. (PDF 33 kb)

Additional file 4: Alkaline phosphatase-based development of whole-mount in situ hybridization in *H. microstoma*: SFRPs and posterior Wnt components. Bars: 50 μm. (PDF 5034 kb)

Additional file 5: Alkaline phosphatase-based development of whole-mount in situ hybridization in *H. microstoma*: Wnt2 and Wnt5. Bars: 50 μm. (PDF 1653 kb)

Additional file 6: List of genes studied in this work. The GeneDB codes (www.genedb.org) of *E. multilocularis* and *H. microstoma* gene models are given, together with the primer sequences used for transcript amplification by RT-PCR. (XLSX 12 kb)

Abbreviations

AP: Antero-posterior; EdU: 5-ethynyl-2′-deoxyuridine; PCG: Position control gene; TPM: Tropomyosin; WMISH: Whole-mount in situ hybridization.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

UK conceived of the study, performed all experiments in *E. multilocularis*, and drafted the manuscript. FJ performed experiments in *H. microstoma*. PDO and KB participated in the design and supervision of the study and helped draft the manuscript. All authors read and approved the final manuscript.

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